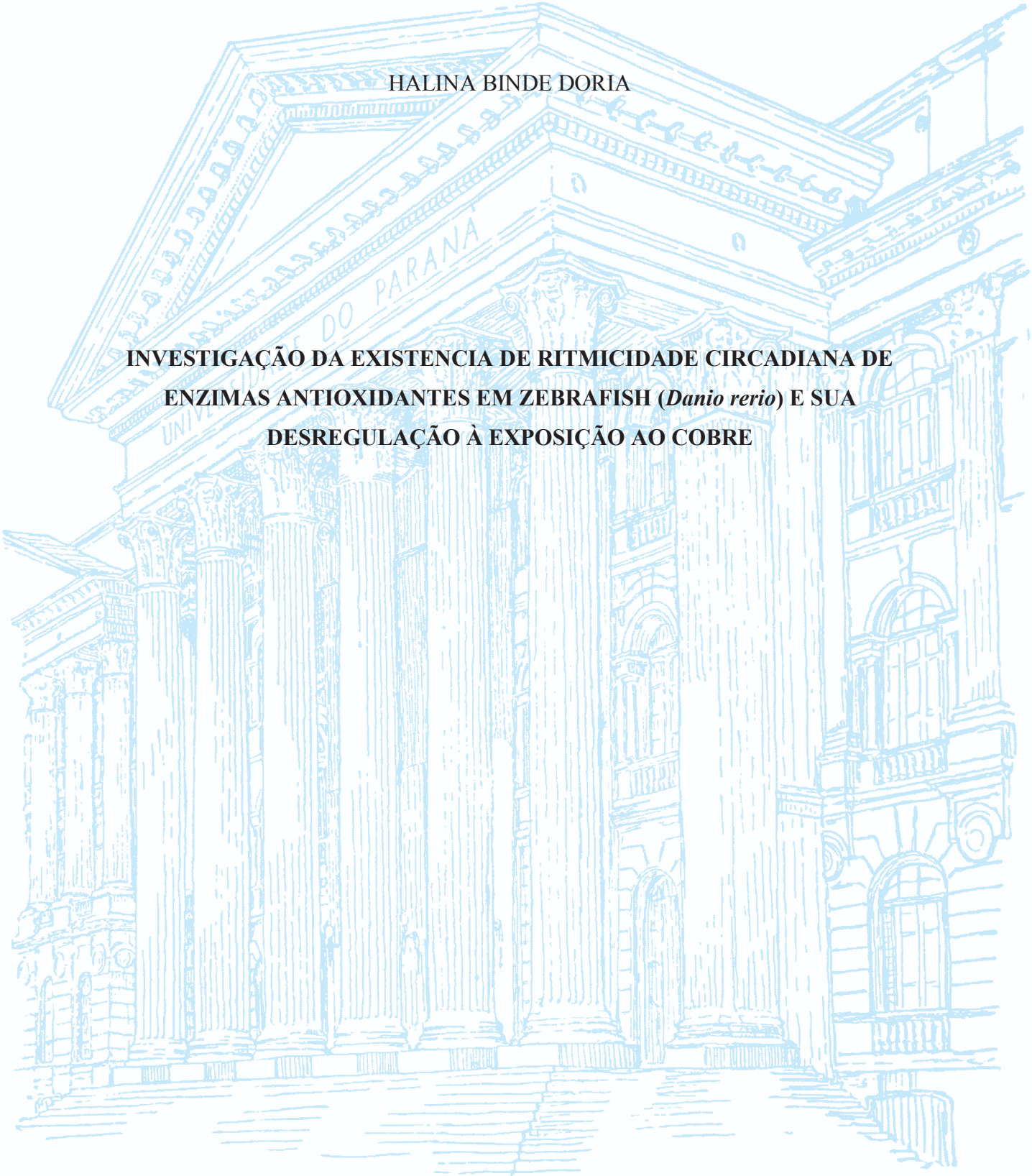
UNIVERSIDADE FEDERAL DO PARANÁ

HALINA BINDE DORIA



INVESTIGAÇÃO DA EXISTENCIA DE RITMICIDADE CIRCADIANA DE ENZIMAS ANTIOXIDANTES EM ZEBRAFISH (*Danio rerio*) E SUA DESREGULAÇÃO À EXPOSIÇÃO AO COBRE

CURITIBA, 2018

UNIVERSIDADE FERDERAL DO PARANÁ

HALINA BINDE DORIA

INVESTIGAÇÃO DA EXISTENCIA DE RITMICIDADE CIRCADIANA DE ENZIMAS ANTIOXIDANTES EM ZEBRAFISH (*Danio rerio*) E SUA DESREGULAÇÃO À EXPOSIÇÃO AO COBRE

Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, Departamento de Biologia Celular, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção de título de doutora.

Orientador: Prof. Dr. Marco Antonio Ferreira Randi

Co-orientador: Prof. Dr. Nicholas Simon Foulkes

CURITIBA, 2018

Universidade Federal do Paraná. Sistema de Bibliotecas.
Biblioteca de Ciências Biológicas
(Telma Terezinha Stresser de Assis –CRB/9-944)

Doria , Halina Binde
Investigação da existência de ritmicidade circadiana de enzimas antixiodantes em zebrafish (*Danio rerio*) e sua desregulação à exposição ao cobre. / Halina Binde Doria. – Curitiba, 2018.
122 f. : il. ; 30cm.

Orientador: Marco Antonio Ferreira Randi
Co-orientador: Nicholas Simon Foulkes
Tese (Doutorado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular e Molecular.

1. Peixe. 2. Sulfato de Cobre. 3. Ritmicidade. I. Título II. Randi, Marco Antonio Ferreira. III. Foulkes, Nicholas Simon. IV. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular e Molecular.

CDD (20. ed.) 574.1882

MINISTÉRIO DA EDUCAÇÃO
SETOR CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO BIOLOGIA CELULAR E MOLECULAR

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em BIOLOGIA CELULAR E MOLECULAR da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **HALINA BINDE DORIA** intitulada: **Investigação da existência de ritmicidade circadiana de enzimas antioxidantes em zebrafish (Danio rerio) e sua desregulação à exposição ao cobre**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 26 de Fevereiro de 2018.

MARCO ANTONIO FERREIRA RANDI
Presidente da Banca Examinadora

LUCELIA DONATTI
Avaliador Interno

FERNANDO MAZZILLI LOUZADA
Avaliador Interno

RODRIGO DE CASSIO DA SILVA
Avaliador Externo

GUSTAVO SOUZA SANTOS
Avaliador Externo

Dedico esse trabalho ao meu avô Newton Doria, que desde minha graduação me incentivou a estudar e sempre quis me ver doutora, e à minha avó Rita Ferri Binde, que preencheu meus dias de comidas deliciosas, conversas malucas e muita alegria. Mesmo que não estejam mais entre nós, não há um dia que passe que eu não me lembre de vocês.

AGRADECIMENTOS

Ao meu orientador Prof. Marco Antonio Ferreira Randi, pela paciência, apoio e por aceitar a ideia desse projeto e confiar no meu trabalho;

Ao meu co-orientador na Alemanha Prof. Nicholas Simon Foulkes, por ter me recebido tão bem no grupo, ter acreditado na minha capacidade e de sempre ter um horário para me ajudar;

A Marianna Boia Ferreira, minha amiga desde que entramos no mestrado, que dedicou muito do seu tempo para me ensinar tudo que podia sobre primers, PCR e cultivo celular;

A Drª. Daniela Vallone por todo ensinamento sobre biologia molecular e ritmos, pela ajuda na bancada e entusiasmo com meus resultados;

Ao Haiyu, Rima, Gennaro, Andrea e Giuseppe pela ajuda no laboratório e principalmente pela companhia e amizade durante meu período no KIT;

As amigas do Laboratório de Toxicologia Celular: Carola, Flávia, Débora, Yvanna, Nilce e Maristela e todos os demais. Obrigada por toda ajuda nas coletas e amizade durante esses anos;

Ao Leonardo, meu namorado, pela ajuda nos fins de semana em que tive que ir para o laboratório, na configuração das figuras da tese e, principalmente pelo companheirismo, carinho e os constantes incentivos ao longo desse último ano;

À Renata, Francine, Janaína e Cauhana, as melhores amigas que a vida me deu, que sempre me ouviram, apoiaram e acreditaram em mim. Obrigada pelas conversas, risadas, desabafos e por sempre estarem ao meu lado não importando o que aconteça. Vocês são minha inspiração para que eu seja uma pessoa melhor a cada dia;

A Prof.ª Lia Sumie Nakao, Silvia Rodrigues e Sze Mei Lo, do departamento de Patologia Básica da UFPR pela colaboração nas análises de quantificação de ATP;

À Cinthia Eloise Domingues, do departamento de química da UEPG pela colaboração com as análises de metais na água;

À secretária do programa de Pós-Graduação em Biologia Celular e Molecular, Marlene e Lucas;

Ao CNPq pela bolsa de doutorado no Brasil e ao DAAD pela bolsa de doutorado sanduíche na Alemanha, em especial à Maria José Salgado Martinez, responsável pelos bolsistas DAAD brasileiros, que sempre foi muito solícita e simpática;

E gostaria de agradecer especialmente aos meus pais, Kátia e Newton, pelo amor e apoio incondicional, por me ensinarem o valor dos estudos e sempre, desde os dias mais felizes até os mais frustrantes, me ouvirem e me guiarem pelo melhor caminho. Meu amor por vocês é imensurável!

RESUMO

Todos os seres vivos possuem um sistema marcador de tempo que é sincronizável com o ambiente onde vivem. À vista disso, uma estreita ligação entre o ritmo circadiano e processos fisiológicos relacionados à produção de espécies reativas de oxigênio (EROs) e de enzimas antioxidantes é relatada em diversos organismos. Por sua vez, o sulfato de cobre ($CuSO_4$) é um composto conhecidamente pró-oxidante e amplamente utilizado em aquicultura como agente terapêutico em peixes. Ele é frequentemente utilizado em concentrações que chegam a ser de sete até cem vezes maiores do que as encontradas em ambientes com influência de atividades antrópicas. Apesar de muitos estudos terem demonstrado o potencial tóxico do sulfato de cobre em diversos organismos aquáticos, a investigação das consequências da exposição a esse metal, e consequente promoção de estresse oxidativo, no ritmo circadiano ainda não foi examinada. Nesta tese, foi avaliado o efeito e possíveis mecanismos moleculares envolvidos na resposta celular das defesas antioxidantes e de proteínas chaves envolvidas na marcação da ritmicidade circadiana em peixes *Danio rerio* e na linhagem celular derivada de embriões da mesma espécie (PAC-2) expostos ao $CuSO_4$. No primeiro capítulo, estudos bioquímicos e de expressão gênica, juntamente com análises químicas indicaram que a exposição hídrica ao $CuSO_4$ interferiu com a coordenação entre as enzimas SOD e CAT e perturbou a oscilação diária de proteínas de controle do relógio. Ainda, evidências concretas sobre a existência de ritmicidade na expressão e atividade de SOD e CAT foram encontradas. Estes resultados indicaram que a sincronização do organismo com o ambiente pode ser prejudicada devido à exposição aguda ao sulfato de cobre. Desta forma, o segundo capítulo foi conduzido de modo a explorar de maneira mais aprofundada as bases moleculares do *cross-talk* entre o cobre, as enzimas antioxidantes SOD e CAT e o relógio circadiano. Com a utilização de técnicas de clonagem molecular, transfecções e quantificação de expressão gênica, os resultados obtidos apoiam fortemente uma relação entre exposição aguda ao Cu, ativação da defesa antioxidante e distúrbios na expressão de genes envolvidos no controle do relógio circadiano através da ativação da via de sinalização da MAPK em células PAC-2. Além disso, também foi demonstrado que a transcrição da SOD pode ser regulada tanto em resposta ao estresse oxidativo como também associada ao relógio circadiano. De maneira geral, este estudo fornece as primeiras informações a respeito da interação toxicológica entre exposição ao Cu e desregulação do mecanismo de controle do relógio circadiano em organismos aquáticos.

Palavras chave: zebrafish, sulfato de cobre, defesa antioxidante, ritmicidade circadiano

ABSTRACT

All living beings present a time-marking system that can synchronize with the environment where they live. A close link between the circadian rhythm and physiological processes related to the production of reactive oxygen species (ROS) and antioxidant enzymes is reported in several organisms. Copper sulfate ($CuSO_4$), in turn, is a compound known as pro-oxidant and widely used in aquaculture as a therapeutic agent in fish. It is frequently applied in concentrations between seven to one hundred times greater than as found in environments with influence of anthropic activities. Although many studies have demonstrated the toxic potential of copper ions in various aquatic organisms, an investigation of the consequences of $CuSO_4$ exposure, consequent promotion of oxidative stress, and the circadian rhythm has not yet been examined. In this thesis, we evaluated the effect and possible molecular mechanisms involved in the cellular response of antioxidant defenses and key proteins involved in circadian rhythm in *Danio rerio* and in the cell line, PAC-2, derived from embryos of the same species, exposed to $CuSO_4$. In the first chapter, biochemical and gene expression studies along with chemical analyzes indicated that exposure to $CuSO_4$ interfered with the coordination between SOD and CAT enzymes and disrupted the daily oscillation of circadian rhythm control proteins. Moreover, concrete evidence on the existence of rhythmicity in the expression and activity of SOD and CAT were found. These results indicated that a synchronization of the organism with the environment may be impaired due to acute exposure to Cu. In this way, the second chapter was conducted in order to explore the molecular bases of the cross-talk between copper, SOD and CAT antioxidant enzymes and the circadian clock. With the use of molecular cloning techniques, transfections and gene expression, the results obtained support a relationship between Cu exposure, activation of the antioxidant defense and disorders in the expression of circadian clock control genes through the activation of the MAPK pathway in PAC-2. It has also been shown that the transcription of SOD can be regulated both as a response to oxidative stress and also associated with the circadian clock. In general, this study provides the first information about the toxicological interaction between Cu exposure and deregulation of the circadian clock control mechanism in aquatic organisms.

Key words: zebrafish, copper sulfate, antioxidant defense, circadian rhythm

LISTA DE FIGURAS

INTRODUÇÃO

FIGURA 1: Modelo de funcionamento da ritmicidade circadiana..... 13
FIGURA 2: Funcionamento molecular básico de controle da ritmicidade circadiana... 14
FIGURA 3: Organização e hierarquia entre oscilador central e osciladores periféricos 16
FIGURA 4: Detoxificação de ânion superóxido. 19
FIGURA 5: Exemplar de *Danio rerio* selvagem adulto..... 22

CAPÍTULO 1

FIGURE 1: Biological responses of clock genes expression in *Danio rerio* liver..... 37
FIGURE 2: Biological responses of clock gene expression in *Danio rerio* brain 38
FIGURE 3: Biological responses of antioxidant defense and energetic cost in *Danio rerio* liver..... 40
FIGURE 4: Biological responses of antioxidant defense in *Danio rerio* brain..... 41
FIGURE 5: Biochemical biomarkers in *Danio rerio* liver and brain..... 42

CAPÍTULO 2

FIGURE 1: MTT, induction of antioxidant response and endogenous expression of sod after treatment with Cu. 75
FIGURE 2: Analysis of – 953sod1-Luc promoter..... 77
FIGURE 3: Activation of the MAPK via ROS production after Cu exposure..... 79
FIGURE 4: Endogenous expression of clock and light regulated genes after Cu exposure 81
FIGURE 5: Rhythms of clock and light-regulated genes after Cu exposure 82

LISTA DE TABELAS

CAPÍTULO 1

TABLE 1: Primers used in the qRT-PCR and their bibliographic source..... 33

CAPÍTULO 2

TABLE 1: Primers used in the qRT-PCR 71

LISTA DAS PRINCIPAIS ABREVIATURAS E SIGLAS

ARE - *antioxidant responsive element*

ATP - *adenosine triphosphate*

CAT - catalase

CRY - *cryptochrome*

Cu - cobre

DBP - *D-box–binding protein*

DCF-DA - *2',7' – dichlorofluorescin diacetate*

DD - *dark/dark*

EROs - espécies reativas de oxigênio

E4BP4 - *E4-binding protein 4*

GPX - glutationa-peroxidase

GR - glutationa reduzida

HLF - *hepatic leukemia factor*

HPLC - *high performance liquid chromatography*

LD - *light/dark*

LRR - *light responsive region*

MAPK - *mitogen-activated protein kinase*

MTT - *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*

PER - *period*

pERK - *phospho-extracellular signal-regulated kinase*

pJNK - *phosphor-c-Jun NH_2-terminal kinase*

pp38 - *phospho-p38 MAPK*

qRT-PCR - *quantitative real-time polymerase chain reaction*

RNA - *ribonucleic acid*

ROS - *reactive oxygen species*

SOD - superóxido-dismutase

TEF - *thyrotroph embryonic factor*

VBP - *vitellogenin gene–binding protein*

XRE - *xenobiotic responsive element*

SUMÁRIO

1. INTRODUÇÃO 12
1.1. RITMICIDADE CIRDADIANA 12
1.1.1. O FUNCIONAMENTO MOLECULAR DA RITMICIDADE CIRCADIANA 13
1.1.2. MAMÍFEROS E PEIXES: MODELOS QUE DIFEREM ENTRE SI 15
1.2. ESPÉCIES REATIVAS DE OXIGÊNIO: ESTRESSE OXIDATIVO E SINALIZAÇÃO CELULAR 17
1.2.1. SOD 19
1.2.2. CAT 20
1.2.3. ESPÉCIES REATIVAS DE OXIGÊNIO E A RITMICIDADE CIRCADIANA 20
1.3. ORGANISMO MODELO: ZEBRAFISH (*Danio rerio*) 21
1.4. COBRE 22
2. JUSTIFICATIVA 23
3. OBJETIVOS 24
3.1. OBJETIVO GERAL 24
3.2. OBJETIVOS ESPECÍFICOS: CAPÍTULO 1 24
3.3. OBJETIVOS ESPECÍFICOS: CAPÍTULO 2 25
4. ESTRATÉGIA EXPERIMENTAL 26
5. CAPÍTULO 1 28
6. CAPÍTULO 2 67
7. DISCUSSÃO GERAL E CONSIDERAÇÕES FINAIS 96
8. BIBLIOGRAFIA GERAL 100
9. RESULTADOS ADICIONAIS 118

1. INTRODUÇÃO

1.1. RITMICIDADE CIRDADIANA

Desde o início, o planeta tem sido submetido a ritmos diários de luz e escuro e também a ciclos sazonais de mudanças climáticas, causadas pela rotação da Terra em torno do seu eixo e em torno do Sol, respectivamente. Portanto, desde o aparecimento da vida, a alternância do dia e da noite deixou marcas significativas sobre os organismos vivos (KULCZYKOWSKA et al., 2010).

Convenientemente, a quase totalidade dos seres vivos, desde os unicelulares até mamíferos e plantas, exibem marcadores de tempo – em inglês, *timekeepers* – endógenos. A marcação de fase pelos *timekeepers* se dá por meio de oscilações rítmicas, dentro do período de 24 horas, de certas proteínas chave no processo da ritmicidade circadiano. A característica básica dos ritmos biológicos é a de persistir sob condições ambientais constantes, na ausência de qualquer estímulo externo direto. Porém, mesmo que os ritmos endógenos difiram daqueles no ambiente natural, sinais de sincronização exógenos - conhecidos como *timegivers* em inglês ou *zeitgeber* em alemão - ajustam a percepção do tempo biológico, de modo que os organismos permanecem em fase com o meio em que vivem (FIGURA 1). Para a maioria das espécies já estudadas, o *zeitgeber* mais importante da ritmicidade circadiana é a variação diária de claridade e escuridão.

Esse tipo de organização temporal endógena torna possível a adaptação e antecipação a ciclos ambientais da natureza e proporciona coordenação temporal para atividades fisiológica e comportamentais (KULCZYKOWSKA et al., 2010; ZELINSKI et al., 2014).

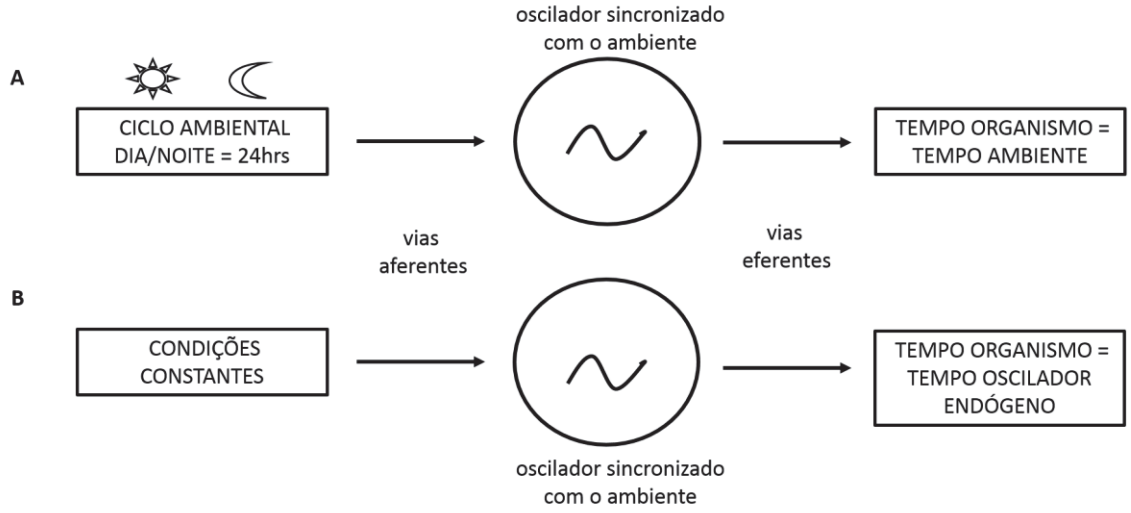


FIGURA 1: Modelo de funcionamento da ritmicidade circadiana. A - sincronizado com ciclos ambientais: O sinal externo (zeitgeber) é levado ao relógio endógeno por vias aferentes; esse, uma vez sincronizado, passa a informação para o resto do organismo por vias eferentes, gerando ritmos biológicos sincronizados em sua fisiologia geral. B - Sistema em livre-curso: expressa o período endógeno próprio. FONTE: modificado a partir de CECON & FLÔRES (2010).

1.1.1. O FUNCIONAMENTO MOLECULAR DA RITMICIDADE CIRCADIANA

Verificou-se que o cerne do relógio circadiano consiste em três alças principais de retroalimentação interligadas que ciclam com um período aproximado de 24 horas (ALBRECHT, 2004; REPPERT & WEAVER, 2002). A primeira consiste em elementos positivos (CLOCK e BMAL1), membros da família de fatores de transcrição que apresentam o domínio bHLH-PAS - em inglês *basic helix-loop-helix - Period-ARNT-Singleminded*. A presença do domínio PAS nas proteínas permite que elas interajam entre si formando heterodímeros, enquanto que o domínio bHLH lhes garante a capacidade, enquanto dímeros, de se ligar a E-boxes presentes na região promotora de diversos genes, entre eles os period (per) e cryptochrome (cry) regulando suas transcrições (CECON & FLÔRES, 2010; VATINE et al., 2011; IDDA et al., 2012).

Após a tradução, a dimerização, e a translocação para o núcleo, das proteínas CRY e PER, elas inibem a ação do heterodímero CLOCK:BMAL1, diminuindo então as transcrições dos genes cry e per. Como consequência, os níveis de mRNA e proteína desses decrescem até o ponto em que tornam-se insuficientes para reprimir a atividade de CLOCK:BMAL1, reiniciando um novo ciclo.

A segunda alça consiste na expressão rítmica do transcrito BMAL1 (EMERY & REPPERT, 2004) e ela é iniciada também por CLOCK:BMAL, que ativa a transcrição dos genes rev-erb e ror, cujas proteínas competem entre si pela ligação aos elementos responsivos ao ROR, presentes no promotor de bmal, onde apresentam ações antagônicas: ROR ativa a transcrição de bmal1 enquanto que REV-ERB a inibe (KO & TAKAHASHI, 2006). Além disso, os promotores de clock e cry1 também apresentam sequências ROREs (CECON & FLÔRES, 2010; IDDA et al., 2012).

A terceira alça por sua vez envolve os fatores de transcrição positivos DPB/ HLF/ TEF, já descritos por terem sua expressão induzidas pela luz (WEGER et al., 2011) e negativo E4BP4. Eles podem atuar ligando-se a sequências D-box nos promotores de alguns genes centrais do controle circadiano, incluindo per e cry (HASTINGS et al., 2007). Essa alça está envolvida na regulação da expressão induzida pela luz, pois sequencias D-box servem como um dos principais elementos responsivos à luz (MRACEK et al., 2012). A presença destas duas alças, juntamente com a manutenção firmemente regulada da primeira, ocorre em fases consecutivas que, em conjunto, contribuem para conferir robustez e precisão sobre o mecanismo do relógio (FIGURA 2).

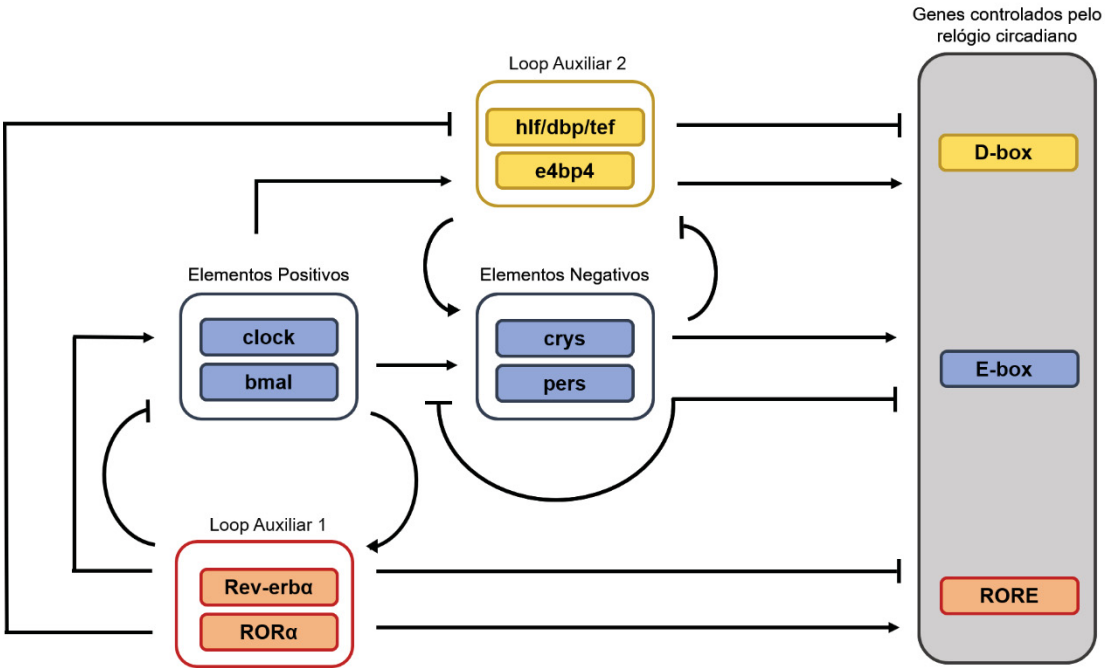


FIGURA 2: Funcionamento molecular básico de controle da ritmicidade circadiana. Molecularmente, a ritmicidade circadiana é regulada por um loop de transcrição auto oscilante. O heterodímero CLOCK/BMAL1 liga-se a regiões E-box localizadas no promotor e estimula expressão de fatores de transcrição essenciais pers e crys. Por sua vez, esses últimos reprimem a atividade transcricional do CLOCK/BMAL1 pela inibição da sua ligação com o E-box localizado em seus próprios promotores através da formação de um complexo com subsequente fosforilação. Os loops auxiliares ajudam na estabilização do loop principal regulador. FONTE: a autora.

1.1.2. MAMÍFEROS E PEIXES: MODELOS QUE DIFEREM ENTRE SI

Inicialmente acreditava-se na existência de um "relógio mestre" altamente centralizado que, em mamíferos, foi identificado como o núcleo supraquiasmático – em inglês *suprachiasmatic nucleus*, SCN – ele funcionava como um tipo de marca-passo circadiano central, sendo que fotorreceptores nos olhos percebiam e transmitiam o estímulo luminoso até ele para o ajuste entre ambiente e tempo endógeno. Em peixes, répteis e pássaros o marca-passo circadiano mostrava-se presente nos olhos e na glândula pineal, sendo ambos diretamente responsivos à luz (TAMAI et al., 2005).

Entretanto, evidências de descentralização do controle da ritmicidade circadiana começaram a surgir em vertebrados não mamíferos (PLAUTZ et al., 1997; BALSALOBRE et al., 1998) e a ideia de um oscilador central composto apenas de poucas estruturas concentradas foi contestada. Supõe-se que a falta de osciladores periféricos diretamente arrastáveis pela luz em mamíferos se deve a que os mecanismos ancestrais de resposta à luz foram convertidos para responderem a outros tipos de sinalização. Isso provavelmente ocorreu devido a condições de falta de luminosidade e o comportamento noturno adotado pelos primeiros mamíferos no início da história evolutiva do grupo (VATINE et al., 2011).

Dessa forma, mesmo que o sistema de temporização circadiana esteja presente em todos os níveis de organização de um organismo e auxilie na demanda crescente de osciladores periféricos específicos para diferentes funções fisiológicas (PANDO & SASSONI-CORSI, 2002; KULCZYKOWSKA et al., 2010) a autonomia desse sistema difere entre espécies (FIGURA 3). Em mamíferos, os osciladores periféricos não são diretamente excitáveis pela luz, mas podem ser estimulados por outros sinais internos como hormônios. Por essa razão, esses animais apresentam maior dependência do oscilador central presente no encéfalo e no SCN para a transmissão de informação sobre os ciclos de claro/escuro, além de apresentar um atraso maior na percepção desse estímulo quando comparados com organismos que exibem osciladores periféricos fotossensíveis (KULCZYKOWSKA et al., 2010; VATINE et al., 2011).

Os peixes, em especial o *Danio rerio*, apresentam osciladores periféricos fotossensíveis e com alto grau de independência do oscilador central. Como exemplo, diferentes tecidos – coração, rins, intestino, nadadeiras, retina, cérebro e fígado - dessa

espécie são diretamente estimuláveis pela luz (WHITMORE et al., 2000; CARR & WHITMORE, 2005). Parâmetros dos osciladores, como a duração do período, resposta à luz e compensação de temperatura variam de tecido para tecido, possivelmente refletindo padrões de expressão diferencial da família de genes responsável pelo controle da ritmicidade circadiana (IDDA et al., 2012).

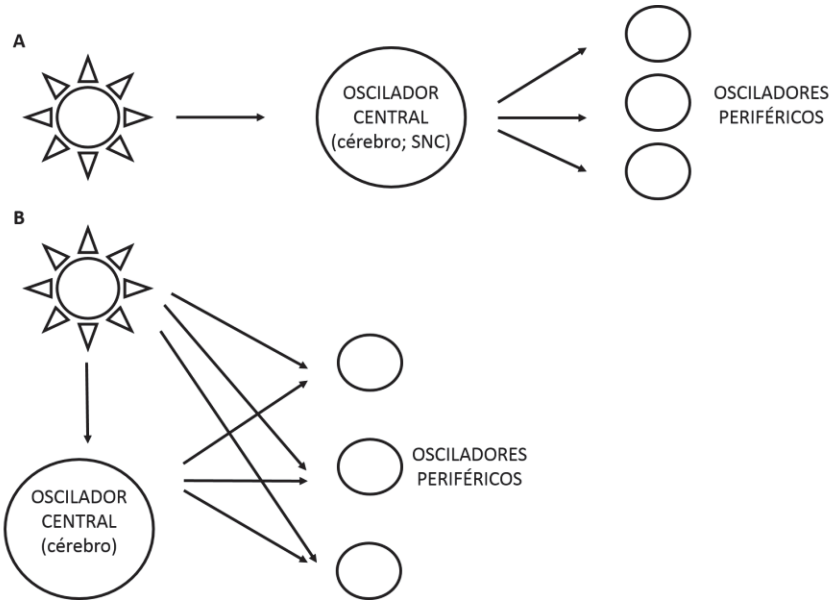


FIGURA 3: Organização e hierarquia entre oscilador central e osciladores periféricos. A- modelo de organização sugerido para mamíferos; B- modelo de organização sugerido para Danio rerio FONTE: modificado a partir de KULCZYKOWSKA et al. (2010).

Entretanto, ainda não se sabe quais são esses fotorreceptores amplamente distribuídos pelos tecidos e de que forma acontece a sinalização nos osciladores periféricos. Porém, atualmente três principais hipóteses existem:

- Opsinas extra-retinais: genes similares aos de opsinas são amplamente expressos em diversos tecidos de vertebrados não mamíferos e poderiam estar envolvidos na percepção e transdução do sinal luminoso, uma vez que essas proteínas foram relatadas como fotorreceptores (CAVALLARI et al., 2011)

- Genes cry: o papel desempenhado pelo CRY como fotorreceptor em neurônios laterais de *Drosophila* e a maior similaridade com um dos homólogos do cry do *Danio rerio*, comparado com o dos mamíferos, fortalece essa hipótese (KOBAYASHI et al., 2000).

- Produção de peróxido de hidrogênio por uma oxidase fototransdutora contendo flavina: HIRAYAMA et al. (2007) observou acumulo de H_2O_2 na linhagem celular Z3 após exposição a comprimentos de onda próximos ao azul-violeta. Isto resultou na

ativação da via de sinalização MAPK, que por sua vez acionou a transcrição dependente da luz dos genes cry1a e per2. A luz também induziu a expressão de catalase que serviu para regular negativamente a resposta à luz, uma vez que essa decompõe o peróxido de hidrogênio em água e oxigênio.

Ainda, há outra diferença entre o controle da ritmicidade circadiana em mamíferos e peixes, pois durante a história evolutiva dos teleósteos todo seu genoma foi duplicado e, em consequência desse evento e da perda gênica diferencial, existem cópias extras dos genes-chave do controle da ritmicidade circadiana em peixes em comparação com mamíferos. Assim, o estudo molecular da maquinaria circadiana em peixes torna-se mais complexo uma vez que, por exemplo, até a data, quatro per, seis cry, três clock e três Bmal foram clonados no *Danio rerio*. Além do mais, os diferentes homólogos extras produzem níveis sutis de controle e regulação da ritmicidade que não são observados em modelos mamíferos (WANG, 2008; IDDA et al., 2012).

Torna-se importante ressaltar que todos os genes per e cry apresentam oscilação robusta sob condições de claro-escuro, porém a expressão oscilatória de per2 e cry1a não acontecem em condições escuro-escuro. PANDO & SASSONI-CORSI (2002) observaram, experimentalmente, que a expressão de per1 e per3 é ativada antes de luzes serem acesas, concluindo que eles são capazes de antecipar o amanhecer e também demonstrando que a expressão per2 e cry1a são estritamente luz-dependente.

1.2. ESPÉCIES REATIVAS DE OXIGÊNIO: ESTRESSE OXIDATIVO E SINALIZAÇÃO CELULAR

O oxigênio é fundamental para os organismos aeróbios, pois é utilizado na transdução de energia através da cadeia transportadora de elétrons na mitocôndria dos eucariotos, ou na membrana celular de muitas bactérias, além da essencialidade em várias outras vias metabólicas. Porém, seu consumo gera espécies reativas de oxigênio (EROs), que compreendem espécies não radicais, como o peróxido de hidrogênio, e radicais livres que são moléculas que possuem um ou mais elétrons desemparelhados. Isso ocorre porque quando o oxigênio molecular consumido pela célula é reduzido a água, acoplado à oxidação de alimentos e transformação de energia como, por exemplo, durante o transporte de elétrons, sua redução pode ser parcial. Isso resulta na formação de EROs

(NORDBERG & ARNÉR, 2001; LIVINGSTONE, 2001; HALLIWELL & GUTTERIDGE, 2007).

As EROs são continuamente produzidas pelo metabolismo celular normal e desempenham papéis fisiológicos importantes na sinalização intracelular, regulação do estado redox (NORDBERG & ARNÉR, 2001), controle da ventilação respiratória, apoptose, diferenciação e proliferação celular, aderência de leucócitos a células endoteliais ou mesmo na ativação da resposta imunológica específica contra patógenos no processo inflamatório (DRÖGE, 2002). Mais recentemente foi descrito, em *Danio rerio*, o controle da ritmicidade circadiana pelo peróxido de hidrogênio e da enzima que o degrada, a catalase (HIRAYAMA et al., 2007).

As EROs têm efeitos diversos sobre as células e se em pequenas quantidades são indispensáveis para a manutenção celular; porém, quando a taxa de produção delas eleva-se pode-se originar o estresse oxidativo, definido como uma perturbação na homeostase do organismo, resultado das ações de estímulos intrínsecos ou extrínsecos, comumente definidos como estressores (WENDELAAR BONGA, 1997; LIVINGSTONE, 2001; PATEL et al., 2014). Quando essa situação acontece as EROs, por serem muito reativas, podem reagir com proteínas, lipídios, outras moléculas celulares ou até mesmo interferir na ativação/inibição de diversas vias de sinalização, prejudicando o funcionamento normal da célula. Vários mecanismos de defesa celular existem para prevenir os possíveis danos causados por EROs e a manter seus níveis dentro do normal. Elas podem ser neutralizadas ou degradadas pelos organismos por defesas antioxidantes como a vitamina C e a glutationa reduzida (GR), ou por enzimas antioxidantes específicas como superóxido-dismutase (SOD), catalase (CAT) e glutationa-peroxidase (GPX). Uma via de detoxificação de ROS é apresentada na FIGURA 4.

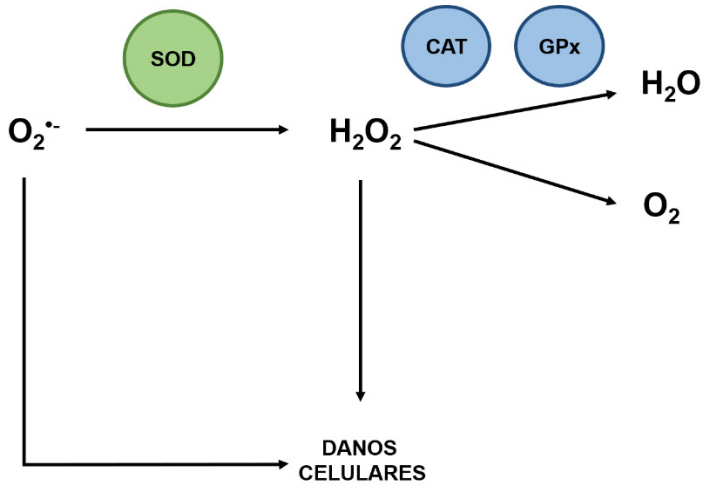


FIGURA 4: Detoxificação de ânion superóxido. O O_2^* gerado pelas células é convertido em peróxido de hidrogênio pela SOD. Na sequência, a CAT, a GPx ou o sistema antioxidantes dependente de glutationa continuam a redução do H_2O_2. FONTE: A autora.

1.2.1. SOD

A SOD é uma metaloenzima que desempenha papel antioxidante central e tem grande importância, pois é observada em todos os organismos aeróbicos. Age sobre o radical do ânion superóxido (O^{-2}) dismutando-o a H_2O_2 (VAN DER OOST et al., 2003; HALLIWELL & GUTTERIDGE, 2007).

$$2O^{-2} + 2H^{+} \longrightarrow O_2 + H_2O_2$$

Foram identificadas três isoformas diferentes de SOD e existem em diferentes compartimentos celulares: Mn-SOD ou SOD2 é encontrada na matriz mitocondrial e as outras duas isoformas possuem Cu e Zn em seu centro catalítico, SOD3 ou EC-SOD está localizada de forma extracelular e SOD1 ou Cu/Zn-SOD é principalmente encontrada no citosol, mas também está presente no núcleo, lisossomos e mitocôndrias (ZELKO et al., 2002; STORZ, 2011; MILANI et al., 2011).

Em diversos estudos foi demonstrado que atividade dessa enzima pode ser usada para mensuração de efeitos adversos resultantes da exposição a compostos que induzem a produção de EROs (LI et al., 2013; SIMONATO et al., 2016). Nesses casos, a atividade da SOD é induzida em poucas horas após a exposição, especialmente se a produção de EROs não for muito exacerbada (VAN DER OOST et al., 2003).

1.2.2. CAT

As catalases são enzimas que tem um grupamento heme e estão localizadas principalmente nos peroxissomos de células eucarióticas. Elas catalisam a eliminação de peróxido de hidrogênio (H_2O_2), liberando como produtos água e oxigênio molecular (VAN DER OOST et al, 2003). A reação predominante depende da concentração de doadores de H e da concentração ou taxa de produção de H_2O_2 no sistema. Entretanto, enquanto a reação peroxidativa é relativamente lenta, a decomposição do H_2O_2 ocorre rapidamente (AEBI, 1984; VENTURA, 2004)

$$2\ H_2O_2 \xrightarrow{\text{CATALASE}} 2H_2O + O_2$$

As catalases também atuam na detoxificação de diferentes substratos na oxidação de doadores de H, como fenóis e álcoois, via redução acoplada de peróxido de hidrogênio:

$$H_2O_2 + R'\ H_2 \longrightarrow R' + 2H_2O$$

Uma das funções antioxidantes da catalase é reduzir o risco da formação do radical hidroxil a partir da H_2O_2 via reação de Fenton, catalisada por íons metálicos de cobre e ferro, principalmente (NORDBERG & ARNÉR, 2001).

1.2.3. ESPÉCIES REATIVAS DE OXIGÊNIO E A RITMICIDADE CIRCADIANA

A partir de dados obtidos em diferentes organismos, incluindo humanos, pode-se observar uma estreita ligação entre o sistema circadiano e processos fisiológicos de sinalização redox celular. Isso muito provavelmente ocorre porque há ritmos nas atividades diárias de um organismo, tais como alimentação, locomoção, atividade

cerebral e o ciclo sono/vigília, modulam a intensidade dos processos metabólicos durante o dia (HARDELAND et al., 2003; PATEL et al., 2014). Assim, já que 2-5% de oxigênio inspirado é transitoriamente convertido em ROS, não há surpresa que exista uma estreita relação entre metabolismo e geração de ROS que resultam em padrões temporais correspondentes de formação de oxidantes (HARDELAND et al., 2003).

Portanto, para que o organismo se defenda da geração rítmica de oxidantes há uma resposta simétrica e compensatória de indução da defesa antioxidante (HARDELAND et al., 2003; PATEL et al., 2014). Ainda, como a cadeia de detoxificação de ROS é baseada numa sequência em que os produtos de uma reação servem como substratos na reação seguinte (FIGURA 4), a expressão e a atividade de vários componentes do sistema de defesa antioxidante devem ser coordenadas (PATEL et al., 2014). Nesse contexto, ritmos diários de enzimas protetoras são descritos em vários organismos filogeneticamente distantes e diferentes tecidos, mas os detalhes relativos a amplitude e o efeito protetivo são altamente divergentes (HARDELAND et al., 2003). A maioria dos dados referem-se ao balanço redox ou das enzimas GST, GR e GPX, porém variações diárias rítmicas para SOD e CAT, inclusive com o organismo em livre curso são relatadas para ratos, camundongos e galinhas (HARDELAND et al., 2003). Infelizmente, a quantidade de dados e estudos em modelos mamíferos é muito mais abundante, detalhada e clara do que para teleósteos, objeto do presente projeto (HARDELAND et al., 2003; VOLPATO & TRAJANO, 2005; PATEL et al., 2014).

1.3. ORGANISMO MODELO: ZEBRAFISH (*Danio rerio*)

O *Danio rerio*, também conhecido por peixe-zebra ou paulistinha, é um teleósteo pertencente à família Cyprinidae, da ordem Cypriniformes (FIGURA 5). Ele é originário de rios de água doce na Índia e, atualmente, é comum em aquários em todo o mundo. É de fácil manutenção, tem rápido ciclo de vida e os métodos para sua criação em laboratório são bem estabelecidos (HILL et al., 2004). Mas a principal vantagem é a disponibilidade da sequência genética completa aliada ao crescente volume de informações moleculares, gênicas e mutagênicas (HILL et al., 2004; VATINE et al., 2011; BRIGGS, 2002).

O interesse inicial no peixe-zebra como um sistema modelo de vertebrados não veio com a necessidade do estudo da ritmicidade circadiana, mas a partir do campo da embriologia e biologia do desenvolvimento (VATINE et al., 2011). Porém, como o sistema circadiano de teleósteos recebeu pouca atenção no decorrer dos anos, o *Danio rerio*, levando-se em conta as vantagens descritas acima, tornou-se o organismo modelo mais apropriado para observação e estudo da ritmicidade circadiana em peixes e a comparação com outros vertebrados (CAHILL et al., 2002; VATINE et al., 2011).

FIGURA 5: Exemplar de *Danio rerio* selvagem adulto. Quando adulto pode chegar até 5 cm, em cativeiro é normal que alcance no máximo 3 cm. FONTE: internet.

Ainda, as linhagens celulares derivadas de embriões do *Danio rerio* apresentam grande potencial para estudo da regulação e função do relógio circadiano de vertebrados (VALLONE et al., 2007). Elas exibem ritmos que podem ser estabelecidos com a simples exposição das células a ciclos de luz-escuro (WHITMORE et al., 2000). Em contrapartida, as linhagens de células de mamíferos requerem tratamentos com serum ou ativadores de vias de sinalização para o início de ritmos transitórios e rápidos. As linhagens derivadas do *Danio rerio* apresentam condições de cultura bastante simples, sendo que crescem à temperatura ambiente, são viáveis durante longos períodos em alta confluência e não requerem uma atmosfera enriquecida em CO_2 (VALLONE et al., 2007).

1.4. COBRE

O cobre é amplamente distribuído na natureza e ocorre naturalmente em muitos minerais (PEDROZO & LIMA, 2001). Ele é também um elemento traço essencial aos organismos, estando presente em um grande número de enzimas envolvidas numa variedade de processos biológicos, mas, quando presente em excesso, pode tornar-se tóxico (SIMONATO et al., 2016).

A exposição ao cobre é inevitável uma vez que ele é um metal abundante na crosta terrestre e o mais antigo metal manipulado pelo homem (NAVARRO, 2006). Mesmo por

fontes naturais, a erosão e lixiviação liberam quantidades significantes em forma de poeira que se depositam na superfície terrestre e em águas superficiais, principalmente. Adicional a isso, o cobre sob a forma de sulfato de cobre ($CuSO_4$) é um composto amplamente utilizado em aquicultura como agente terapêutico em peixes, para controle de protozoários, fungos, bactérias e cianobactérias (VARO et al., 2007; STRAUS & TUCKER, 1993). Embora possam existir alternativas até mais eficientes e seguras, em relação a concentração terapêutica, a utilização do sulfato de cobre persiste principalmente pelo aspecto econômico.

Níveis de ocorrência natural de cobre (Cu) variam de 2 a 30 μg/L e em áreas com prevalência de atividades agropecuárias, mineração e indústrias e efluentes municipais o Cu pode estar presentes em concentrações tóxicas que variam de 50 a 560 μg/L. Entretanto, as concentrações de sulfato de cobre utilizadas na aquicultura são bastante altas e amplamente variáveis, sendo encontrados relatos de 4 mg/L utilizado como bactericida (MACFARLANE et al., 1986) até 6000 mg/L como antifúngico (PAQUIN et al., 2002). No entanto, no Brasil, o CONAMA (2005) define o nível máximo permitido para o cobre dissolvido em águas destinadas ao abastecimento humano (Classe II) como 9 μg/L.

Os íons de cobre apresentam alta reatividade ao H_2O_2 e potencial para sofrer reações redox que formam EROs, um processo conhecido como reação de Fenton. Esses radicais são responsáveis por exercer a função tóxica do excesso de Cu através de quadros de estresse oxidativo. Os danos causados pelas ROS podem alterar a permeabilidade das membranas, a estrutura da cromatina, a síntese de proteínas e várias outras atividades enzimáticas (VALKO et al., 2005; HELSEL & FRANZ, 2015).

Semelhante a outros organismos, os peixes combatem o aumento na geração de ROS com, por exemplo, enzimas antioxidantes como a SOD e a CAT, que convertem ânions superóxido em H_2O_2 e em H_2O e O_2, respectivamente (CRAIG et al., 2007).

2. JUSTIFICATIVA

Os estudos publicados sobre o sistema circadiano em peixe-zebra concentraram-se na caracterização de comportamentos e ritmos fisiológicos, na distribuição dos

osciladores e fotorreceptores em diferentes tecidos, no desenvolvimento embrionário da ritmicidade circadiana e nos padrões de expressão e funções dos genes homólogos do peixe-zebra em comparação com os invertebrados e vertebrados. Porém, pouco se sabe sobre a relação entre produção de espécies reativas de oxigênio, sistema antioxidante e a ritmicidade circadiana em peixes teleósteos.

Novas evidências, como a hipótese do controle da expressão de proteínas chave da ritmicidade circadiana pela enzima catalase em *Danio rerio* (HIRAYAMA et al., 2007) sugerem que as enzimas antioxidantes podem estar intimamente ligadas a componentes do ritmo circadiano endógeno. Essa nova evidência exige que mais pesquisas sejam conduzidas para, não apenas a consolidação do conhecimento, mas também a posterior geração de dados mais sólidos que ajudem na aplicação destes parâmetros em outras áreas como a toxicologia.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar o efeito e possíveis mecanismos moleculares envolvidos na resposta celular das defesas antioxidantes e de proteínas chaves envolvidas na marcação da ritmicidade circadiana em peixes *Danio rerio* e na linhagem celular derivada de embriões da mesma espécie, PAC-2, expostos ao sulfato de cobre.

3.2. OBJETIVOS ESPECÍFICOS: CAPÍTULO 1

- Expor peixes *Danio rerio* a três diferentes concentrações de sulfato de cobre (5 μg/L, 25 μg/L e 45 μg/L), avaliando entre 48 e 72 horas de exposição os efeitos do $CuSO_4$ em fígado e encéfalo sobre:

    - a atividade enzimática e expressão das enzimas SOD e CAT;
    - a expressão de per1, per2 e cry1a;
    - o custo energético do organismo;

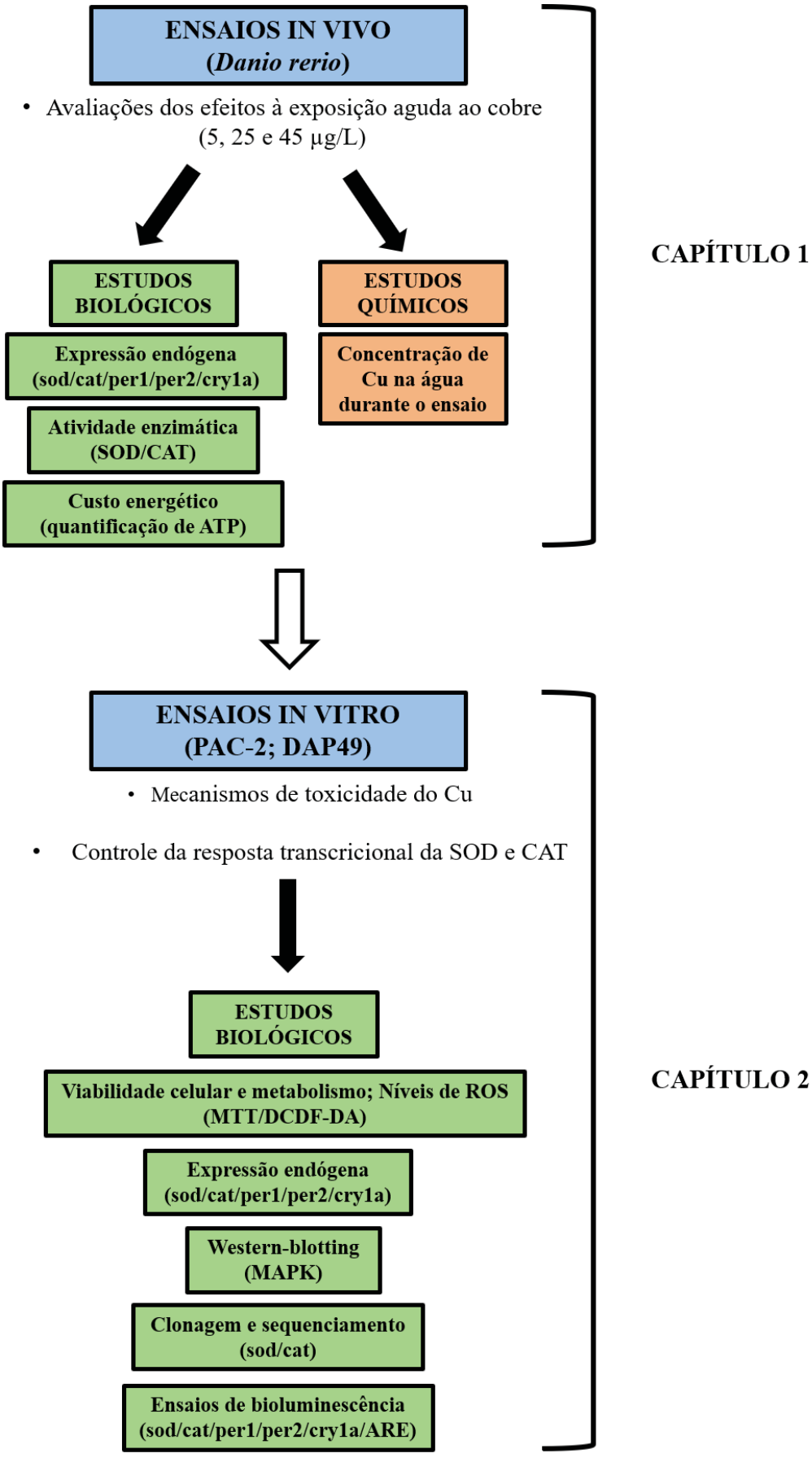
- a correlação da expressão e atividade das enzimas antioxidantes SOD e CAT com a expressão de per1, per2 e cry1a, responsáveis pela marcação de fase endógena.

3.3. OBJETIVOS ESPECÍFICOS: CAPÍTULO 2

- Expor a linhagem de células derivadas de embrião de *Danio rerio* PAC-2 a uma concentração de sulfato de cobre (250 μM) avaliando:

  - as bases moleculares do *cross-talk* entre o cobre, a luz, as enzimas antioxidantes e o relógio circadiano;
  - os mecanismos subjacentes à resposta transcricional da expressão da SOD e CAT;
  - a influência do sulfato de cobre na expressão e sincronização de genes regulados pela luz.

4. ESTRATÉGIA EXPERIMENTAL


ENSAIOS IN VIVO
(Danio rerio)
• Avaliações dos efeitos à exposição aguda ao cobre (5, 25 e 45 µg/L)
ESTUDOS BIOLÓGICOS
Expressão endógena (sod/cat/per1/per2/cry1a)
Atividade enzimática (SOD/CAT)
Custo energético (quantificação de ATP)
ESTUDOS QUÍMICOS
Concentração de Cu na água durante o ensaio
CAPÍTULO 1
ENSAIOS IN VITRO
(PAC-2; DAP49)
• Mecanismos de toxicidade do Cu
• Controle da resposta transcricional da SOD e CAT
ESTUDOS BIOLÓGICOS
Viabilidade celular e metabolismo; Níveis de ROS (MTT/DCDF-DA)
Expressão endógena (sod/cat/per1/per2/cry1a)
Western-blotting (MAPK)
Clonagem e sequenciamento (sod/cat)
Ensaios de bioluminescência (sod/cat/per1/per2/cry1a/ARE)
CAPÍTULO 2

5. CAPÍTULO 1

Artigo publicado na Ecotoxicology and Environmental Safety

Time Does Matter! Acute Copper Exposure Abolishes Rhythmicity of Clock Gene in *Danio rerio*

Halina Binde Doria[1], Marianna Boia Ferreira[1], Silvia Daniele Rodrigues[2], Sze Mei Lo[2], Cinthia Eloise Domingues[3], Lia Sumie Nakao[2], Sandro Xavier de Campos[3], Ciro Alberto de Oliveira Ribeiro[1], Marco Antonio Ferreira Randi[1]

[1]Federal University of Paraná (UFPR), Department of Cellular Biology, School of Biological Sciences, Address: Caixa Postal 19.031, Curitiba, Paraná, Brazil, Postal Code: 81531-990.

[2]Federal University of Paraná (UFPR), Department of Basic Pathology, School of Biological Sciences, Address: Caixa Postal 19.031, Curitiba, Paraná, Brazil, Postal Code: 81531-990.

[3]Ponta Grossa State University (UEPG), Research Group on Environmental and Sanitary Analytical Chemistry (QAAS), Address: Caixa Postal 992, Ponta Grossa, Paraná, Brazil, Postal Code: 84030-900

Declarations of interest: none

Abstract

The circadian clock is a key cellular timing system that coordinates physiology and behavior. Light is a key regulator of the clock mechanism via its activation of Per and Cry clock gene expression. Evidence points to a key role of reactive oxygen species (ROS) in resetting this process. In this context, the aim of the present study was to explore copper sulfate as a ROS generator, using an innovative approach investigating its effects on circadian timing. Liver and brain from *Danio rerio* specimens exposed to 0, 5, 25 and 45 μg/L of copper sulfate concentrations were obtained. Daily oscillations of superoxide dismutase (SOD) and catalase (CAT) enzymatic activity and their correlations both with clock genes (per1, per2, and cry1a) and with organism energy cost were determined. CAT expression correlates with per2 and cry1a and, thus, provides data to support the hypothesis of hydrogen peroxide production by a phototransducing flavin-containing oxidase. Higher SOD activity is correlated with higher intracellular ATP levels. Copper disturbed the daily oscillation of antioxidant enzymes and clock genes, with disturbed per1 rhythmicity in both the brain and liver, while cry1a rhythmicity was abolished in the liver at 25 μg/L of copper sulfate. Coordination between the SOD and the CAT enzymes was lost when copper sulfate concentrations exceeded the limits established by international laws. These results indicate that that organism synchronization with the environment may be impaired due to acute copper exposure.

Key words: Antioxidant Enzymes, Circadian Clock, Copper Sulfate, *Danio rerio*

1. Introduction

Organisms, from unicellular species to mammals and plants, display rhythmic oscillations, within the 24-hour period, named circadian rhythms. These are highly conserved endogenous temporal organizations that allow the anticipation of environmental cycles and provide temporal coordination for physiological and behavioral activities (Kulczykowska et al., 2010; Zelinski et al., 2014; Moore & Whitmore, 2014). Molecularly, the vertebrate circadian clock is regulated by self-oscillating transcription translation feedback loops, composed of activator and repressor clock proteins. (Nader et al., 2010; Mracek et al., 2012).

The major environmental timing cue for the entrainment of the circadian clock is light. In this way, organisms have evolved dedicated photoreceptors and signaling pathways at all levels of organization, from cells to tissues and organs, that rely on this lighting information (Pando & Sassoni-Corsi, 2002; Mracek et al., 2012). Fish, especially *Danio rerio*, present peripheral clock oscillators directly entrained by light (Whitmore et al., 2000; Vallone et al., 2004). Molecularly, light can promptly activate the transcription of per2 and cr1a genes, that lead to the entrainment of the circadian clock and consequent organism synchronization with the environment within (Ziv et al., 2005; Tamai et al., 2007; Hirayama et al., 2007).

Additionally, it has been demonstrated that ROS (reactive oxygen species), more specifically H_2O_2, via the MAPK signaling pathway, can activate the transcription of those light-regulated genes and, with a delay, also the catalase enzyme. The activation of the catalase will decompose the hydrogen peroxide and downregulate the cycle (Hirayama et al., 2007). Likewise, because circadian rhythms in daily activities, such as feeding, locomotion, brain activity and sleep/wake cycles, modulate the intensity of metabolic processes during the day (Hardeland et al., 2003; Patel et al., 2014), the close connection between the circadian clock and redox signaling have been already pointed out by several studies (Rutter et al., 2002; Hirayama et al., 2007; Krishnan et al., 2008; Jimenéz-Ortega et al., 2011; O´Neill & Feeny, 2014; Wu & Reddy, 2014).

However, when ROS production rates rise, oxidative stress, a disturbance in organism homeostasis resulting from the actions of intrinsic or extrinsic stressors, can occur (Wendelaar Bonga, 1997; Livingstone, 2001; Patel et al., 2004). Since, it is known that ROS play important physiological roles in intracellular signaling pathways (Nordberg & Arnér, 2001; Dröge, 2002; Wang et al., 2010; Tseng et al., 2012), oxidative stress may disrupt cellular homeostasis. Therefore, to protect the organism against excessive ROS

formation, a symmetric and compensatory induction of the antioxidant defense occurs (Hardeland et al., 2003; Patel et al., 2014). Furthermore, since the ROS detoxification chain is based on a sequence in which the products of one reaction serve as substrates for the next, the expression and activity of various components of the antioxidant defense system must be coordinated (Hardeland et al., 2003; Volpato & Trajano, 2005; Patel et al., 2014). Two important components are the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), which convert superoxide anions into H_2O_2 and H_2O and O_2, respectively (Craig et al., 2007).

Copper (Cu) is the oldest metal manipulated by man and exposure to it is inevitable (Navarro, 2006). Natural Cu levels range from 2 to 30 µg/L but, in areas with a prevalence of agricultural and mining activities, industries and municipal effluents, Cu may be present in concentrations ranging from 50 to 560 µg/L (Hem, 1985; Robins et al., 1997). Furthermore, copper sulfate ($CuSO_4$) is widely used in aquaculture as a therapeutic agent for the control of protozoa, fungi, bacteria and cyanobacteria in fish (Varo et al., 2007; Straus & Tucker, 1993), being frequent in concentrations that reach seven to one hundred times greater than those found in the environment (Macfarlane et al., 1986; Paquin et al., 2002). Therefore, although Cu is an essential trace element in organisms, present in a large number of enzymes involved in a variety of biological processes, in high concentrations it can become toxic (Simonato et al., 2015).

Cu ions shows high reactivity to H_2O_2 and the potential to undergo the Fenton reaction, a process that can directly produce ROS, responsible for exerting the toxic function of excess Cu through oxidative stress. (Valko et al., 2005; Helsel & Franz, 2015). Farther, Cu and consequently ROS production, has been demonstrated to activate several signaling pathways but, mainly, the MAPK (Ostrakhovitch et al., 2002; Mattie et al., 2008; Song & Freedman, 2009; McElwee et al., 2009; Wang et al., 2010; Tseng et al., 2012; Turski et al., 2012). This demonstrates that Cu not only can activate antioxidant defenses and interfere with cellular survival, growth and proliferation and apoptosis control, but also it can potentially affect the control of circadian rhythms by exerting oxidative stress.

Currently, an emerging issue is how the circadian apparatus is adjusted to maintain coordination between physiology and the ever-changing environment (Jiménez-Ortega et al., 2011). In this context, As Cu activates the MAPK pathway by producing ROS, and the fact that CAT may also be involved on the control of circadian rhythmicity, the hypothesis or the present work is that exposure to Cu may not only disturb antioxidant

defenses, but also deregulate the circadian timing and proper synchronization between the organism and the environment. Consequently, the present study aimed to evaluate and establish a correlation between the expression and activity of the antioxidant enzymes SOD and CAT with the expression of per1, per2 and cry1a genes, responsible for the control of circadian rhythms, in liver and brain of *Danio rerio* between 48 and 72 hours after Cu exposure to three different concentrations of copper sulfate (9 µg/L, 45 µg/L and 90 µg/L).

2. Material and methods

2.1.Animals and experimental design

In total, approximately 2500, 1:1 males/females between 4 and 5 months old weighting 275.32±46.29 mg, wild-type zebrafish were obtained from an ornamental fish farm (Lindóia; Muriaé-MG) and were acclimated in 30 L or 20 L glass-aquaria for two weeks prior to the beginning of the experiment. The animals were maintained under controlled temperature (26.5±1.5°C), pH (7±0.2) and lighting conditions of 12:12 light/dark cycles (LD). Fish were fed with ALCON Shrimp twice a day and half of the water in the aquaria was renewed every 72 hours.

Four experimental groups were evaluated: one negative control group (CTRL), with no Cu added to the water, and three groups exposed to two ecologically relevant waterborne $CuSO_4$ concentrations of 5 and 25µg/L and one concentration expected only in areas influenced by anthropogenic sources of 45µg/L. A fresh 1 mg/L of Cu stock solution was prepared from copper sulfate (Sigma; ≥99.99% trace metals basis) and deionized water before animal exposure. Cu was added to the aquaria at 8:00 AM, when the lights went on. Fish sampling began after 72 hours of exposure and at each sampling time, 24 fish were randomly collected from the aquaria. The sampling occurred every three hours, starting at time zero (T0) at 8:00 AM and ending at 5:00 AM the next morning (T21), totaling eight collection points during twenty-four hours. The experiments were performed in independent triplicates. For each replica and for each experimental group consisted in 4 glass-aquaria with stocking density of 2-3 fish/liter, as recommended by the European zebrafish community and the Federation for Laboratory Animal Science Associations (FELASA).

After anesthetization with MS222 (1g/L) liver and brain of all fish were obtained and immediately frozen in liquid N_2 for further analysis of enzymatic activity and ATP quantification by HPLC. For the qRT-PCR assay, tissues were embedded in RNAlater®

and frozen in liquid N_2 until RNA extraction. Each time point evaluated for qRT-PCR or enzyme activity had n= 15 animals, except the HPLC measurement that had n=5. At the first (T0) and last (T21) samplings, a 500 mL water sample was taken from each aquarium and added to acidified polyethylene plastic bottles with ultrapure MERCK nitric acid. The bottles were protected from light with aluminum foil and stored at -4ºC until Cu concentration analyses.

This project complies with national and international ethical standards. All the animal procedures were approved by the Ethics Committee on the Use of Animals of the Biological Sciences Sector of the Federal University of Paraná (CEUA/BIO - UFPR). Certificate Nº. 962; Process No. 23075.118616/2016-33.

2.2.qRT-PCR

Pools of three livers and three brains was used for RNA extraction, based on the TRIzol® method (Chomczynski, 1993). RNA integrity was checked with 1% agarose gel with ethidium bromide at 0.5 µg/ml. The electrophoretic run was performed in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.5). RNA was dosed on a NanoVue (GE Healthcare, UK) and only samples with a 260/280nm ratio above 0.8 were used for the Quantitative real-time PCR analysis (qRT-PCR). The primers for per1, per2, cry1a, cu/zn sod, cat and βactin (Table 1) were ordered from EXXTEND (Campinas, São Paulo, Brazil) and purified by the RP-OPC technique, which excludes truncated sequence oligos. The specificity of each primer pair was verified by normal PCR and real-time PCR.

	Forward	Reverse	Source
per1	ccgtcagtttcgcttttctc	Atgtgcaggctgtagatccc	Weger et al., 2013
per2	gcttcaccacaccatacagg	Gtctgacggggacgagtct	Mracek et al.,2013
cry1a	aggcttacacagcagcatca	Ctgcactgcctctggacttt	Peyric et al., 2013
cu/zn sod	tgagacacgtcggagacc	Tgccgatcactccacagg	Lerebours et al., 2009
cat	cctgtggggcgttttg	Cggtacgggcagttgac	Lerebours et al., 2009
βactin	atggatgaggaaatcgctgcc	Ctccctgatgtctgggtcgtc	Jin et al., 2010

TABLE 1: Primers used in the qRT-PCR and their bibliographic source

The Power SYBR® Green RNA-to-CT 1-Step Kit Kit (Applied Biosystems) was used to evaluate the expression of the genes presented in Table 1. Standardization for 10 µl qRT-PCR reactions, for both liver and brain, comprised 5 ng of total RNA and 800 nM

for all the primers. All samples were run in triplicate and submitted to 45 amplification cycles on an AppliedBiosystems StepOnePlus Real-Time PCR System. For quantification, the ΔCt method was used and β-actin was chosen as the endogenous control.

2.3. *Enzyme activity*

Pool of three livers and three brains was homogenized in Tris-HCl buffer, pH 7.2. The homogenates were centrifuged for 20 minutes at 4 ºC at 12,000 g. Total protein concentrations in aliquots of the supernatants were determined by the Bradford (1976) method, using bovine serum albumin as standard.

SOD activity was determined by the method proposed by Kono et al. (1978), which is based on the ability of this enzyme to inhibit the reduction of NBT to blue formazan by the O_2^- generated by hydroxylamine in an alkaline solution. Samples were normalized to 0.2 mg/ml and 0.5 mg/ml of protein for the liver and the brain, respectively. On a 96-well transparent microplate, 10 µl of the normalized samples, 80 µl of 285 mM NBT 0.05 mM EDTA solution and 110 µl of sodium carbonate buffer (182 mM, pH 10.2) with 67 mM hydroxylamine were added to start the reaction. All solutions were maintained at 27 °C. The readings were obtained at 560 nm on a spectrophotometer with a constant temperature of 27 °C at 1-minute intervals for 30 minutes. Enzymatic activity was expressed as U/mg protein.

CAT activity was determined based on the method described by Aebi (1984), that measures catalase through the consumption of hydrogen peroxide. The analysis was performed in 96-well UV transparent microplates. Samples were normalized to 0.2 mg/ml and 0.5 mg/ml of protein for liver and brain, respectively. In the microplate, 5 µl of the normalized liver samples were added to 215 µl of the reaction medium (2.5 ml of 1.0 mM Tris HCl buffer, 5.0 mM EDTA pH 8.0; 47.32 ml of water; 180 µl H_2O_2). For brain, 10µl of the normalized sample and 210 µl of reaction medium were mixed. Enzymatic activity was expressed in µml/min/mg protein.

2.4.ATP Quantification by HPLC

The protocol was adapted according to Manfredi et al. (2002). Five livers were lysed with 10 µL of 0.4 M perchloric acid per milligram of tissue. The livers were then homogenized with a pestle and left on ice for 30 minutes. The homogenate was centrifuged at 13000g at 4°C for 15 minutes and the pellet was separated for subsequent

protein dosing for normalization. The supernatant was neutralized with 10 µl of 4 M K_2CO_3 per 100µl of supernatant and the samples were kept on ice for 10 minutes and then left at -80 °C for one to two hours. After centrifugation at 13000g at 4°C for 15 minutes the supernatants were filtered at 0.22µm (Millipore). For protein quantification, the pellets were resuspended in 100 µL of 2M guanidine and dosed with the Bradford reagent (Biorad).

The column used was Gemini 5u C18 110 A, 150x4.60 mm (Phenomenex) elute with a flow rate of 0.7 ml/min. Buffer A was composed of 25 mM NaH_2PO_4, 0.29mM tetrabutylammonium, pH 5 and buffer B was composed of 10% (v/v) acetonitrile (Sigma), 200 mM NaH_2PO_4, 0.29 mM tetrabutylammonium, pH 4. The gradient of the method was 100% buffer A on the first 5 minutes, switching to 100% buffer B from 5 to 20 minutes and returning to buffer A for 20 to 35 minutes. ATP was determined by UV detector (at 260 nm). Standard curve with commercial ATP (Sigma) was used for quantification. The retention time of the samples were between 23 and 24 minutes.

2.5.Chemical analysis

The determinations of Cu concentrations in the water were carried out on an atomic absorption spectrometer (Varian®, AA 240Z), with electrothermal atomization in graphite furnace (GTA 120 model), equipped with Zeeman bottom broker and automatic display (PSD model 120). A Cu and Ar hollow cathode lamp was used as inert gas at a flow of 0.3 $L.min^{-1}$ with pyrolysis at 1,400 °C and atomization at 2,600 ° C.

2.6.Statistical analyses

The results of enzyme activity and relative gene expression by qRT-PCR were expressed as means ± standard error of the mean. Data normality was verified by the Kolmogorov-Smirnov test. An analysis of variance (ANOVA) of a pathway followed by the Bonferroni test was used. The decision rule (α) was of 0.05 in all analyses. ATP quantification was qualitatively analyzed. Correlation between the parameters was calculated according to Mukaka et al., (2012). Acro software 3.5 by Dr. Refinetti was used for further analysis of circadian rhythmicity and acrophases of normalized data were determined using the single cosinor procedure.

3. Results

No fish mortality was observed during the experiments. The chemical analysis of inorganic Cu concentrations in the water indicated that the fish were initially exposed to 3.293±0.3671 µg/l, 23.46 ± 2.278 µg/l and 43.93±0.8727 µg/l of Cu at the lowest, intermediate and highest concentrations, respectively. At the end of the experiment, total copper concentrations were 2.492±0.6568 µg/l, 18.63±2.771 µg/l and 35.69 ± 2.810 µg/l, respectively.

The separated graphs for each parameter measured in each tissue and concentration are presented, along with the indication of the statistical differences, on the Appendix 1. Here, due to a matter of space and succinctness, a single graph for all the experimental groups to each parameter analyzed in each tissue is shown.

3.1. Per1

The expression of the per1 gene under control conditions showed rhythmicity in the LD cycle in both liver and brain, with acrophases at 11:36 AM and 8:54AM, respectively. When exposed to the lowest $CuSO_4$ concentration, per1 lost rhythmicity, but maintained peak expression at the T0/T3 in both tissues. In the 25µg/L group, liver per1 expression was rhythmic and displayed acrophases at 10:24 AM and in the brain, per1 acrophase was at 11:54 AM. However, on the 45µg/L group, although per1 expression in liver continued to present circadian rhythmicity its acrophase was at 14:00 (Fig.1a and Fig. 2a).

3.2. Per2

The relative expression of per2 in control conditions and when exposed to the 5µg/L $CuSO_4$ concentration in both tissues did not exhibit rhythmicity, but a sharp increase occurred nine hours and between zero and tree hours after the lights went on in liver and brain, respectively (Fig.1b and Fig.2b). However, when exposed to the intermediate and the higher $CuSO_4$ concentrations, the expression of per2 in liver showed two distinct expression peaks at T3 and T6. In the brain, the 25µg/L group showed higher and sustained per2 expression from T0 until T6 and the 45µg/L group showed higher peak expression at T21, three hours before the lights went on (Fig. 1b and Fig. 2b).

3.3. Cry1a

During control conditions, the expression of the cry1a gene exhibited rhythmicity in the liver, with acrophase at 10:42 and a greater relative expression was found 9 hours after the lights went on in the brain. When fish were exposed to $CuSO_4$ no circadian

rhythmicity was detected in liver or in brain. Although the peak expression in liver after the exposure to the lowest $CuSO_4$ concentration was maintained at T3, the exposure to 25 µg/L and 45 µg/L of $CuSO_4$ in liver showed two peak expressions at T3 and T6. In the brain cry1a expression was altered, in comparison to the control, in all groups exposed to $CuSO_4$. Being, the 25 µg/L and the 45 µg/L groups the ones with most disturbances, having highest cry1a expression at 2:00 PM and at 8:00 AM and 8:00 PM, respectively (Fig.1c and Fig. 2c).

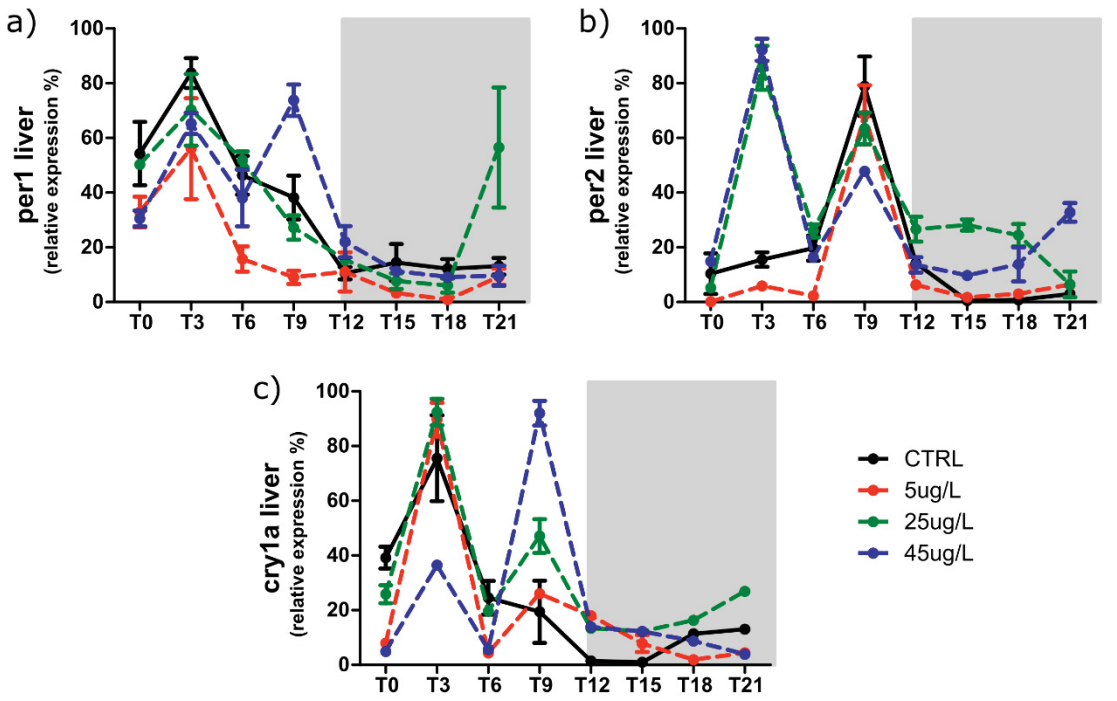


FIGURE 1: Biological responses of clock genes expression in *Danio rerio* liver. The grey area represents the dark period. CRTL- control group; 5 µg/L – fish exposed to the lowest $CuSO_4$ concentration; 25 µg/L- fish exposed to the intermediate $CuSO_4$ concentration; 45 µg/L- fish exposed to the highest $CuSO_4$ concentration. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%). Data are expressed as means ± standard error of the means.

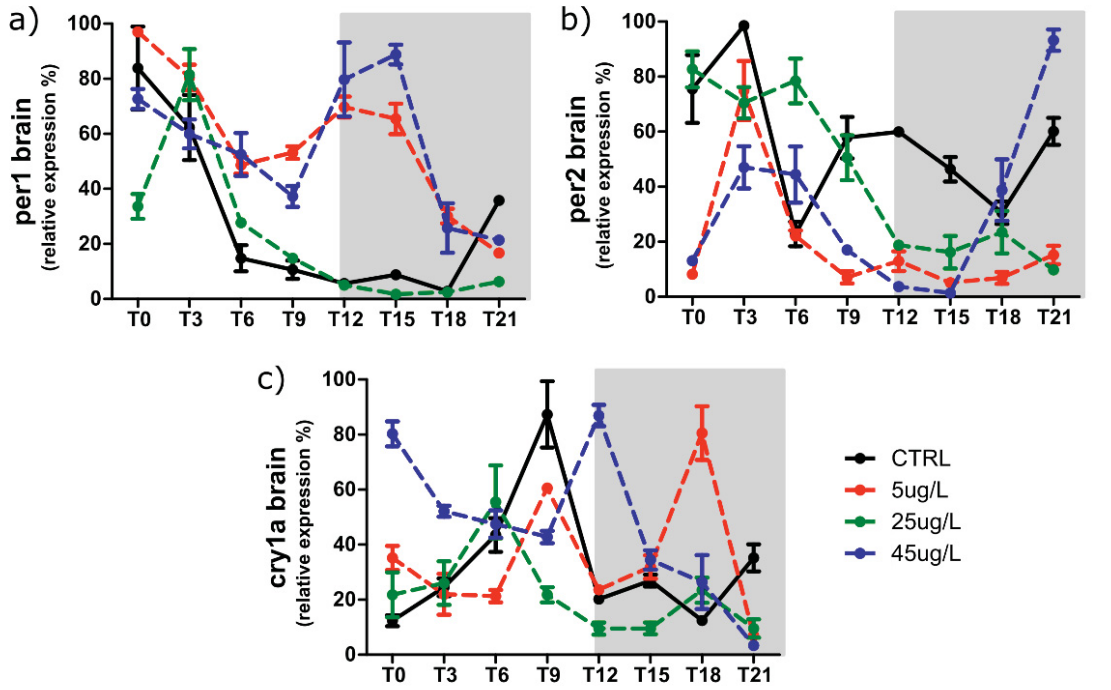


FIGURE 2: Biological responses of clock gene expression in *Danio rerio* brain. The grey area represents the dark period. CRTL- control group; 5 µg/L – fish exposed to the lowest $CuSO_4$ concentration; 25 µg/L- fish exposed to the intermediate $CuSO_4$ concentration; 45 µg/L- fish exposed to the highest $CuSO_4$ concentration. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%). Data are expressed as means ± standard error of the means.

3.4. CAT

CAT showed rhythmic relative expression in control conditions in both the liver and the brain, with acrophases at 9:50 PM and 11:54 PM, respectively (Fig.3a and Fig.4a). Moderate and inversely proportional correlations between cat and per2 expression in the brain (-0.6) and between CAT and cry1a expression in the liver (-0.64) were observed (Mukaka, 2012). Exposure to 5µg/L of $CuSO_4$ result on loss of rhythmicity, but the peak expression in both tissues was similar to the control conditions. The cat expression in liver and brain was higher between 2:00 and 5:00 AM when fish were exposed to the 25 µg/L of $CuSO_4$. But at the higher Cu concentration, no difference in the expression between all sampling times was identified (Fig.3a and Fig.4a).

CAT activity did not display circadian rhythmicity in any of the analyzed tissues, but exhibited feasible ultradian rhythmicity in liver in the control and the 5µg/L groups. In both tissues, two peaks of higher activity, one in the late afternoon and another in the early morning, were detected. However, from the intermediate to the higher $CuSO_4$ concentration CAT activity in both tissues showed evidence of disturbed 24 hours

patterns, being significantly higher between 5:00 to 8:00 AM on the 45 µg/L group (Fig.3b and Fig.4b). Overall analysis of CAT activity in the liver was increased in all exposed groups when compared to the controls (Fig. 5a).

3.5. SOD and ATP quantification

SOD expression under control conditions in the liver showed circadian rhythmicity and acrophase at 7:24 PM (Fig.3c). In the brain, the relative expression presented acrophase at 9:12 AM (Fig.4c). Strong and directly proportional correlations between SOD and per2 expression in the brain (0.749) and liver (0.721) were observed. In turn, when exposed to 5µg/L of $CuSO_4$ SOD expression in the liver and brain did not show any clear rhythms but were higher at 11:00 AM (Fig.3c and Fig.4c). In liver and brain during 25 µg/L and 45 µg/L exposure SOD expression was higher during the light period.

In control conditions and when exposed to the lowest $CuSO_4$ concentration, SOD activity in the liver and the brain and intracellular ATP levels demonstrated probable ultradian patterns (Fig.3c and 3e and Fig.4c). A moderate positive correlation (0.52) was observed between SOD activity in the liver and intracellular ATP levels. But, SOD activity in liver in fish from the 5µg/L group showed a delay of 3 hours of peak activity when compared to the control. While, brain SOD activity in 5µg/L group also showed disturbed pattern in comparison to the control condition. Moreover, SOD activity in liver of the 25 µg/L group showed two peaks of higher activity, at 5:00PM and 2:00AM, also corresponding to higher intracellular ATP content. But this coordination was lost on the 45 µg/L group where SOD activity showed no clear rhythm pattern and intracellular ATP levels could not be measured in the first, third, fifth and last sampling times due to limit of detection of the method.

Finally, when comparing the three $CuSO_4$ concentrations with the controls without taking into account sampling time, a concentration-response pattern could not be identified (Fig.5). SOD activity in liver was higher in the 25ug/L and 45 ug/L Cu-exposed groups when compared to the controls (Fig.5c). In the brain, higher SOD activity was observed only in the group exposed to the highest $CuSO_4$ concentration (Fig.5d). Intracellular ATP levels in the liver were higher in the 5ug/L group compared to the other $CuSO_4$ concentrations (Fig. 5e).

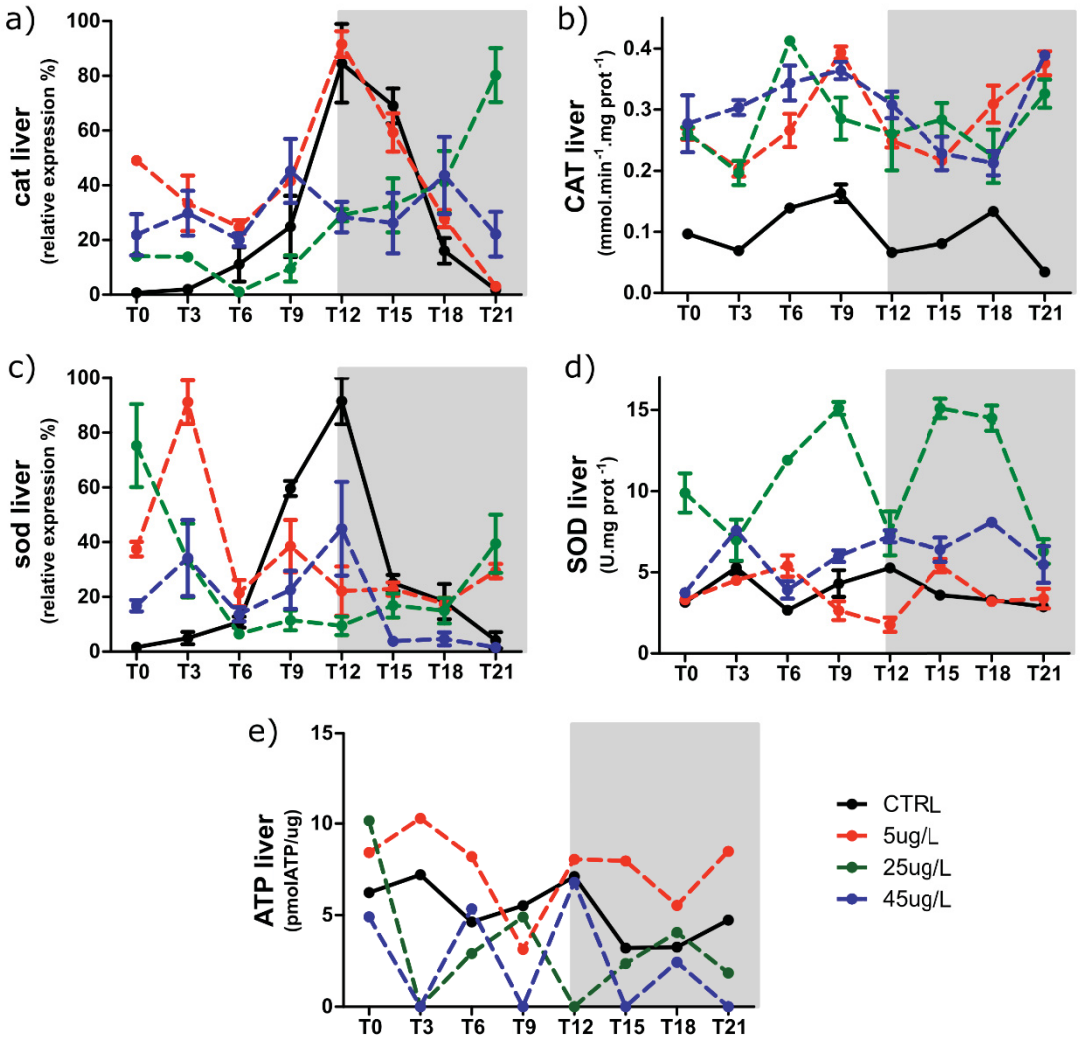


FIGURE 3: Biological responses of antioxidant defense and energetic cost in *Danio rerio* liver. The grey area represents the dark period. CRTL- control group; 5 µg/L – fish exposed to the lowest $CuSO_4$ concentration; 25 µg/L- fish exposed to the intermediate $CuSO_4$ concentration; 45 µg/L- fish exposed to the highest $CuSO_4$ concentration. a) endogenous cat relative expression (%); b) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); c) endogenous sod relative expression (%); d) specific SOD activity (U/hour/mg protein); e) intracellular ATP concentration (pmolATP/ug). Data are expressed as means ± standard error of the means.

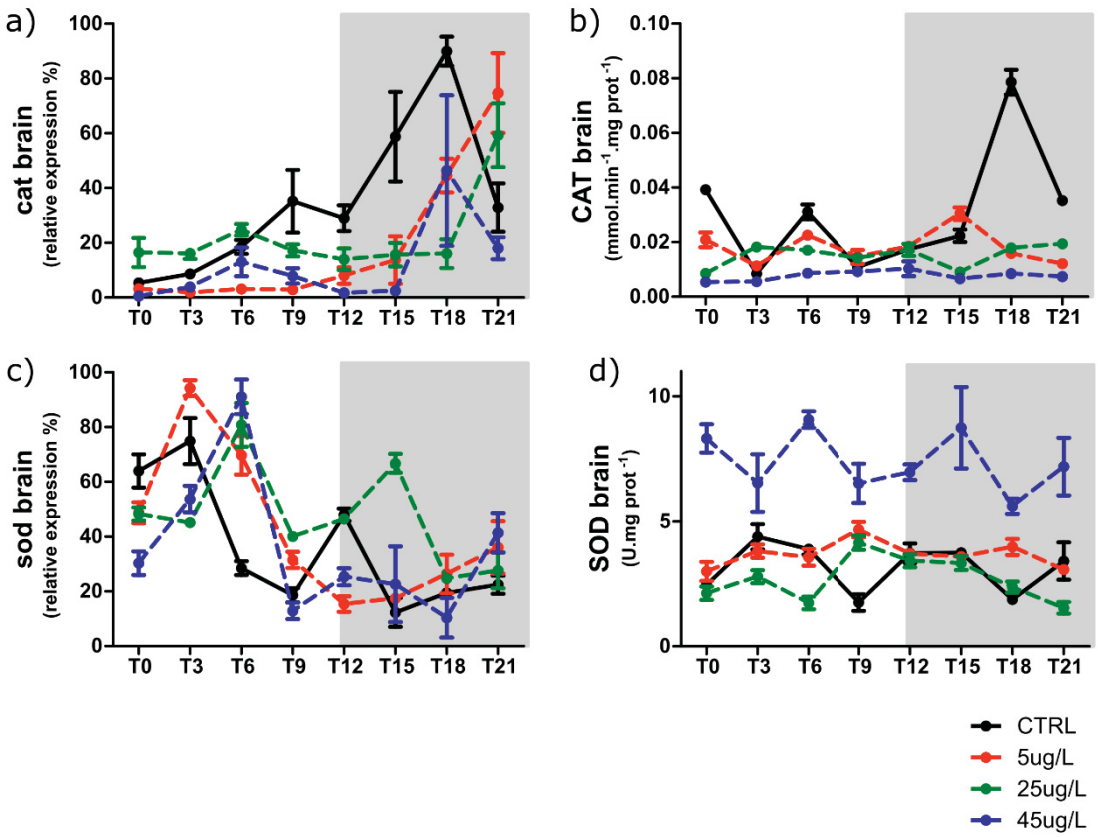


FIGURE 4: Biological responses of antioxidant defense in *Danio rerio* brain. The grey area represents the dark period. CRTL- control group; 5 µg/L – fish exposed to the lowest $CuSO_4$ concentration; 25 µg/L- fish exposed to the intermediate $CuSO_4$ concentration; 45 µg/L- fish exposed to the highest $CuSO_4$ concentration. a) endogenous cat relative expression (%); b) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); c) endogenous sod relative expression (%); d) specific SOD activity (U/hour/mg protein). Data are expressed as means ± standard error of the means.

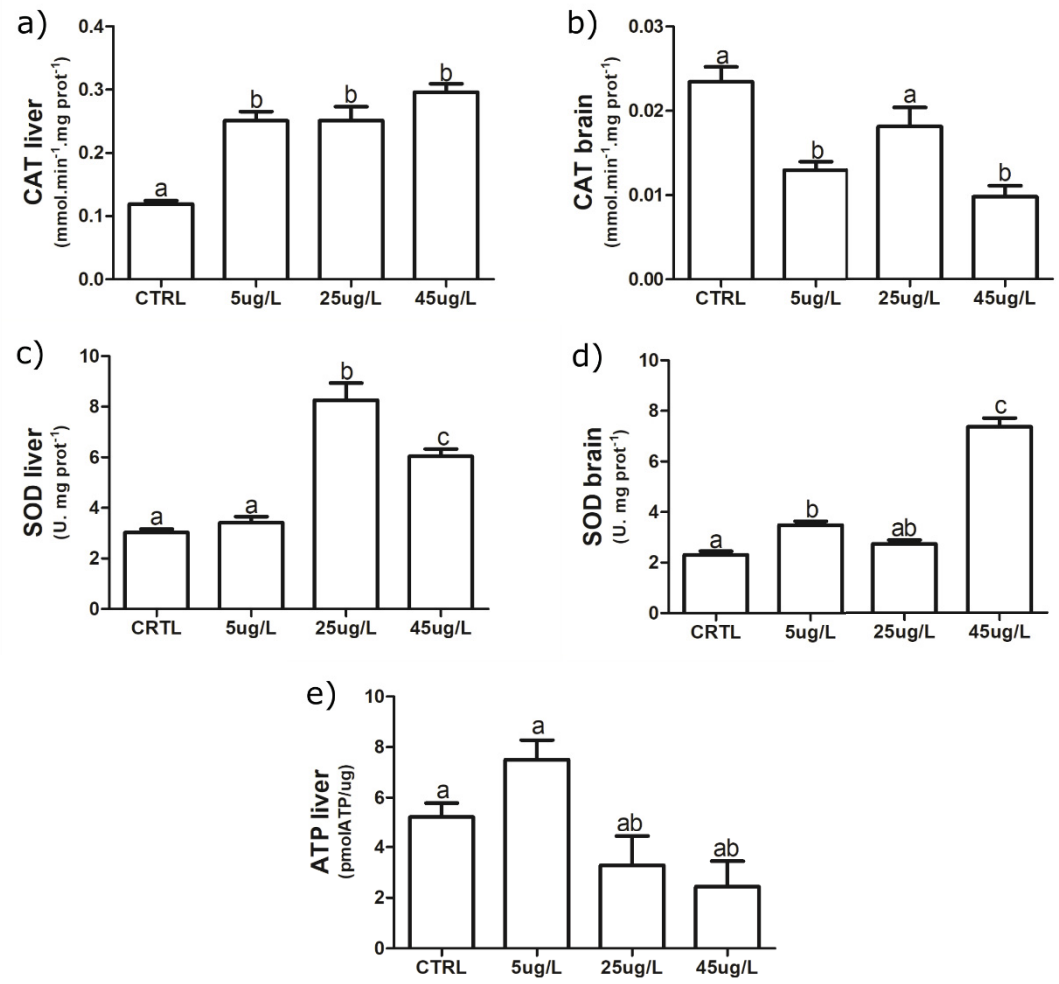


FIGURE 5: Biochemical biomarkers in *Danio rerio* liver and brain. CRTL- control group; 5 µg/L – fish exposed to the lowest $CuSO_4$ concentration; 25 µg/L- fish exposed to the intermediate $CuSO_4$ concentration; 45 µg/L- fish exposed to the highest $CuSO_4$ concentration. a) specific CAT activity in liver (mmol of degraded H_2O_2/min/mg protein); b) specific CAT activity in brain (mmol of degraded H_2O_2/min/mg protein); c) specific SOD activity in liver (U/hour/mg protein); d) specific SOD activity in brain (U/hour/mg protein); e) intracellular ATP concentration in liver (pmolATP/ug). Data are expressed as means ± standard error of the means. Different letters indicate significant differences between groups ($p<0.05$).

4. Discussion

4.1.Expression of per1, per2 and cry1a in control conditions are in accordance with the literature

Agreeing with previous data collected in *Danio rerio* cell cultures (Pando et al., 2001), hypothalamus (Hoskins et al., 2012), muscle tissue (Amaral & Johnston, 2012) and intestinal tissue (Peyric et al., 2013), the expression of the clock regulated gene per1 exhibited a circadian rhythm and had its acrophase in the brain after the lights went on,

and in the liver with a delay of a little more than two hours compared to the brain, demonstrating that the peak expression of per1 in different tissues could vary from the end of the dark phase to the onset of the light period (Velardea et al., 2010; Kulczykowska et al., 2010; López-Olmeda et al. 2009).

Per2 and cry1a are light-regulated genes (Tamai et al., 2007; Mracek et al., 2012) and deletions of D-box and E-box in the gene promoters generate loss of light responsiveness (Vatine et al., 2009). Thus, there is usually a transient and abrupt increase in their expressions, often not resulting in rhythmic patterns (Mracek et al., 2013 Ben-Moshe et al., 2014), which was observed in the present study. In addition, as reported for *Carassius auratus*, where the peak expression of per2 in the gut preceded the retina (Velardea et al., 2010), a peak expression of per2 in the brain preceded the peak in the hepatic tissue, while the opposite occurred for cry1a expression. As it is known that the various pers and crys copies operates at different control and regulation levels (Wang, 2008; Idda et al., 2012) and that per2 acts in association with cry1a as elements of the light pathway, it is possible to conceive of an offsetting expression of these genes in different tissues.

4.2.Higher SOD activity correlated with higher levels of intracellular ATP

SOD is the first line of defense against the superoxide radical (Van der Oost et al., 2003; Halliwell & Gutteridge, 2007) and daily patterns in enzymatic activity have been reported in several organisms (Albarrán et al., 2001; Hardeland et al., 2003; Martin et al., 2003; Goncharova et al., 2006; Subramanian et al., 2008).

In the present study, the pattern of SOD expression in liver was similar to that described for rat liver by Xu et al. (2012). In turn, SOD activity in *Danio rerio* liver and brain under LD control conditions showed strong evidence of ultradian rhythmicity, which, however, cannot be asserted given the experimental design adopted herein. In

addition, a similar SOD activity pattern was observed in *Macaca mulata* erythrocytes, which displayed lower and higher SOD activity at the approximate times found for *Danio rerio* liver and brain (Goncharova et al., 2006).

It has been suggested that these variations in antioxidant enzyme activity may protect the organism from excessive ROS production and damage to macromolecules (Hardeland et al., 2003; Kondratov, 2007). Therefore, these patterns can be correlated to feeding, brain activity for locomotor activity cycles, and, consequently, higher ROS production. This scenario is corroborated by two peaks of intracellular ATP, indicative of higher activity and energetic cost for the organism, which overlapped with higher SOD activity. Thus, it can be concluded that SOD activity is directly influenced by the energy cost of the organ.

4.3.*CAT expression correlated with per2 and cry1a expression, corroborating the hypothesis of the control of the circadian rhythm by hydrogen peroxide*

In control conditions, two higher CAT activity peaks in the liver, six hours and eighteen hours after the light went on, are consistent with previous results from rat liver (Xu et al., 2012; Sani et al., 2006) and may also be related to the existence of an ultradian rhythm. In the brain, peak activity seventeen hours after the lights turned on was equally found in rat brain (Sani et al., 2006).

Furthermore, the relative CAT expression in the liver oscillated in the antiphase for cry1a and, in the brain, in the antiphase for per2. This data, in light of the previous study conducted by Hirayama et al. (2007), supports the hypothesis that CAT is closely linked to the circadian rhythm control of light-regulated genes. In addition, this supports that this occurs not only in cell cultures, but also in distinct organs.

Interestingly, SOD expression can also be positively correlated with per2 in the brain and the liver. Thus, these results point to the need for further research into different control mechanisms between antioxidant enzymes and clock- and light- regulated genes.

4.4.It was not possible to establish a correlation between expression and enzymatic activity, although coordination between SOD and CAT activity is clear.

It is known that an interconnection between transcription levels and antioxidant enzymes activity is not always clear, either because of the extensive post-translational control of these enzymes or because they are slow but long-acting enzymes (Xu et al.2012). Nevertheless, in the present study, it was noticeable that peaks of CAT activity were preceded by higher SOD activity in the control group, demonstrating a joint action of these enzymes in neutralizing ROS.

However, when fish were exposed to the intermediate and highest $CuSO_4$ concentrations, this coordination was not observed any longer. Thus, as the coordination between SOD and CAT activities is indispensable for the perfect functioning of the antioxidant enzymatic system, it can be inferred that exposure to a pro-oxidant, such as $CuSO_4$, and likely, the generation of oxidative stress and damage to macromolecules, can be caused first by the decoupling of the action of the first line of enzymatic defense of the antioxidant cycle.

4.5.Acute exposure to low $CuSO_4$ concentrations did not have a clear effect on the expression of the clock and light regulated genes, but activated the antioxidant system

Fish exposed to 5µg/L $CuSO_4$ showed similar peaks when compared to the controls, both in the liver and in the brain, for per1, per2 and cry1a gene expressions. Likewise, a

high similarity of CAT expression and activity patterns compared to the control fish was observed. However, the correlations found for cat and per2 expressions in the brain and cat and cry1a expressions in the liver were weaker. However, this does not show significant influence on the expression of the clock and light regulated genes and, consequently, the synchronization between the environment and the fish.

In the liver, the highest SOD expression peak was advanced in three hours and lost its circadian rhythmicity in relation to the control. Thus, disturbances in the twenty-four hours activity pattern were observed in both organs, liver and brain. This can be explained, at least for the hepatic tissue, by the correlation between higher levels of intracellular ATP combined with higher SOD activity.

Thus, acute $CuSO_4$ exposure can contribute to a small increase in the energy cost of the organism and activate antioxidant enzyme defenses, first, by altering the twenty-four hour SOD activity pattern and, subsequently, by altering the overall CAT activity, higher in the liver and lower in the brain when compared to the controls. These results confirm previous research proposing that low Cu concentrations in freshwater, ranging from 2 to 8 ug/L, are already sufficient to activate biological responses and lead to adverse effects (Karan et al., 1998; GRNC, 2002; Craig et al., 2007; Mela et al., 2013; Simonato et al., 2016; Silva Acosta et al., 2016).

4.6.Environmentally relevant $CuSO_4$ concentrations disturbed the twenty-four hour pattern of antioxidant enzymes and light-regulated genes

Light is a key stimulus for the synchronization of the circadian clock. It induces the production of ROS that, in turn, acts in photoreception (Hirayama et al., 2007). Thus, higher ROS levels produced by Cu exposure via the Fenton reaction (Valko et al., 2007) and disturbances in the activity of antioxidant enzymes may interfere with the expression

of the per2 and cry1a genes. Furthermore, it is known that ROS can activate the MAPK pathway that is essential in the light-induced transcriptional activation of clock genes cry1a and per2 (Cermakian et al., 2002; Hirayama et al., 2009; Ramos et al., 2014) and also for the activation of antioxidant defenses via ARE/Nrf2 pathway (Shi and Zhou, 2010; Chen et al., 2017; Jeong et al., 2017).

Accordingly, changes in per2 and cry1a expression in liver and brain, as well as in CAT and SOD activity and expression patterns compared to the negative control, were detected. However, in agreement with the hypothesis that CAT represses the expression of light-regulated genes, per1 rhythmicity was not altered in the liver and the brain. Thus, even if this represents an environmentally relevant condition, the intermediate Cu concentration is above of that recommended by USEPA (1984) for the protection of aquatic fauna, of 20 ug/L.

4.7.per1 lost rhythmicity when fish were exposed to the highest Cu concentration

When exposed to 45ug/l of $CuSO_4$, all the parameters evaluated herein were altered. The rhythm of per1 in the brain was lost and, in the liver, acrophase was delayed by two and a half hours. Furthermore, the similarity of per2 and cry1a expression patterns in liver of fish exposed to intermediate and high $CuSO_4$ concentrations persisted, with two distinct higher expression peaks. The production pattern of the intracellular ATP concentrations, as well as the expression and activity of SOD and CAT, were drastically disturbed, suggesting loss of energetic homeostasis and a stress situation.

Thus, since all genes, both light and clock regulated ones, displayed distinct expression when compared to the negative controls, and observing what is described in the literature for *D. rerio* (Velardea et al., 2010, Mracek et al., 2012, Amaral & Johnston,

2012, Peyric et al., 2013), the results imply that synchronization between the organisms and the environment may be impaired.

Behavioral studies in zebrafish exposed to realistic Cu concentrations indicate that concentration as low as 7 ug/l and up to 40 ug/l may reduce animal fitness (Sloman et al., 2003; Vieira et al 2009; Silva Acosta et al., 2016). Increased locomotor activity and memory loss have been observed and may both be correlated with disturbances in the circadian rhythm (Cho et al., 2012; Hur et al., 2012; Silva Acosta et al., 2016). Thus, it is possible to state that acute exposure to $CuSO_4$ above 20 ug/l disturbs the circadian rhythm in liver and the brain, interfering with the correct synchronization between the organism and the environment.

4.8.The higher the $CuSO_4$ concentration, the higher the disturbances

It was possible to establish that disturbances in the rhythms or 24-hour patterns of the evaluated parameters increased with increasing $CuSO_4$ concentrations. However, by comparing the three $CuSO_4$ concentrations without taking into account the sampling time with increasing $CuSO_4$ concentrations does not reveal the concentration-response principle. Another important point to be observed is exposure time, since the daily oscillations of the parameters to be evaluated can have consequences on the results of the chemical exposure at different times of the day (Hooven et al., 2009).

Thus, the present study demonstrates that taking into account the sample collection schedule and the knowledge of the oscillatory patterns of the evaluated parameters is essential for the generation of adequate data in ecotoxicology. In addition, adopting a more holistic view that considers time, according not only to the bioindicator organism, but also to its biomarkers, may be a more efficient alternative. Even if more laborious in

the practical sense, the generation of reliable data must be the goal for the field of ecotoxicology.

4.9.Toxic metals can disturb circadian rhythmicity

Although metals have multiple known effects on biological systems, among them the ability to interfere with the antioxidant defense (Valko et al., 2005; Jaishankar et al., 2014), their role in modulating gene expression is still poorly studied. In addition, very few studies have been devoted to investigating deregulation in circadian rhythms.

In this context, Jiménez-Ortega et al. (2011) have already described cadmium (Cd) as a disruptor of per1, per2 and cry1a rhythms and some antioxidant defense in rats. Both Cd and Cu can undergo redox reactions and, thus, directly generate ROS. However, the primary pathway for Cd toxicity is by depletion of glutathione and binding to sulfhydryl groups of proteins (Valko et al., 2005; Jiménez-Ortega et al., 2011). Rats exposed to cigarette smoke, which is a known pro-oxidant, also displayed altered expression of genes involved in the control of circadian rhythms (Gebel et al., 2006). In addition, exposure to bisphenol-A, an endocrine disruptor and, at low concentrations, an oxidative stress promoter (Zhou et al., 2011; D'Cruz et al., 2012; Hulak et al., 2013), also affects circadian rhythmicity and leads to asynchrony in all examined genes (Rhee et al., 2014)

5. Conclusions

The present study is the first one to show that exposure to environmentally relevant $CuSO_4$ concentrations, that promotes oxidative stress, can impair organism synchronization with the environment by interfering with light and clock-regulated genes, namely cry1a, per2 and per1 genes. On that matter, coordination between SOD and CAT

enzyme activity was lost when $CuSO_4$ concentrations exceeded the permitted by international laws, possibly worsen the oxidative stress situation.

Moreover, it was possible to correlate CAT expression with per2 and cry1a expression, providing data for the consolidation of the hydrogen peroxide production hypothesis by a phototransducing oxidase containing flavin. Thus, more studies on the MAPK pathway and regulation of SOD and CAT expression and activity should be done in order to investigate the biological mechanisms by which exposure to copper sulfate induces cellular antioxidant defense and deregulates the expression of genes linked to circadian rhythm control in *Danio rerio*.

Acknowledgments

This research was supported by CNPq (PhD scholarship). The authors wish to thank Danilo Marchete Damas de Souza and Hernandes Faustino de Carvalho from UNICAMP and Marta Margarete Cestari from UFPR for their technical support.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Aebi, H. Catalase in vitro. Methods in Enzymology. 105:121–126. 1984. doi: 10.1016/S0076-6879(84)05016-3.

Albarrán, M.T.; López-Burillo, S.; Pablos, M.I.; Reiter, R.J.; Agapito, M.T. Endogenous rhythms of melatonin, total antioxidant status and superoxide dismutase activity in several tissues of chick and their inhibition by light. Journal of Pineal Research. 30:227–233. 2001. doi: 10.1034/j.1600-079X.2001.300406.x.

Amaral, I.P.G.; Johnston, I.A. Circadian expression of clock and putative clock-controlled genes in skeletal muscle of the zebrafish. American Journal of Physiology.

Regulatory, Integrative and Comparative Physiology. 302:R193–206. 2012. doi: 10.1152/ajpregu.00367.2011.

Ben-Moshe, Z.; Alon, S.; Mracek, P.; Faigenbloom, L.; Tovin, A.; Vatine, G. D.; Eisenberg, E.; Foulkes, N.S.; Gothilf, Y. The light-induced transcriptome of the zebrafish pineal gland reveals complex regulation of the circadian clockwork by light. Nucleic Acids Research. 42:3750–3767. 2014. doi:10.1093/nar/gkt1359.

Bradford, M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry. 72:248–254. 1976. doi: 10.1016/0003-2697(76)90527-3.

Cermakian, N.; Pando, M.P.; Thompson, C.L.; Pinchak, A.B.; Selby, C.P.; Gutierrez, L.; Wells, D.A.; Cahill, G.M.; Sancar, A.; Sassone-Corsi, P. Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. Current Biology 12: 844-848. 2002. doi: 10.1016/S0960-9822(02)00835-7.

Chen, Z.; Wan, X.; Hou, Q.; Shi, S.; Wang, L.; Chen, P.; Zhu, X.; Zeng, C.; Qin, W.; Zhou, W.; Liu, Z. GADD45B mediates podocyte injury in zebrafish by activating the ROS-GADD45B-p38 pathway. Cell death & disease, 7: e2068. 2017. doi: 10.1038/cddis.2015.300.

Cho, H.; Zhao, X.; Hatori, M.; Yu, R.T.; Barish, G.D.; Lam, M.T.; Chong, L.W.; Di Tacchio, L.; Atkins, A.R.; Glass, C.K.; Liddle, C.; Auwerx, J.; Downes, M.; Panda, S.; Evans, R.M. Regulation of Circadian Behavior and Metabolism by Rev-erbα and Rev-erbβ. Nature. 485:123–127. 2012. doi: 10.1038/nature11048.

Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques. 15:532-534, 536-537. 1993.

Craig, P.M.; Wood, C.M.; Mcclelland, G.B. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 293:1882-1892. 2007. doi: 10.1152/ajpregu.00383.2007.

D'Cruz, S.C.; Jubendradass, R.; Mathur, P.P. Bisphenol A induces oxidative stress and decreases levels of insulin receptor substrate 2 and glucose transporter 8 in rat testis. Reproductive Sciences. 19:163-172. 2012. doi: 10.1177/1933719111415547.

Dröge, W. Free radicals in the physiological control of cell function. Physiological Reviews. 82: 47–95. 2002. doi: 10.1152/physrev.00018.2001.

Gebel, S.; Gerstmayer, B.; Kuhl, P.; Borlak, J.; Meurrens, K.; Muller, T. The kinetics of transcriptomic changes induced by cigarette smoke in rat lungs reveals a specific program of defense, inflammation, and circadian clock gene expression. Toxicological Sciences. 93:422–431. 2006. doi: 10.1093/toxsci/kfl071.

Goncharova, N.D.; Shmaliy, A.V.; Bogatyrenko, T.N.; Koltover, V.K. Correlation between activity of antioxidant enzymes and circadian rhythms of corticosteroids in

Macaca mulatta monkeys of different age. Experimental Gerontology. 41:778–783. 2006. doi: 10.1016/j.exger.2006.04.006.

GRNC - Groupe de Radioecologie du Nord Cotentin. Risque pour l'environnement– Evaluation des risques associes aux rejets chimiques des installations nucleaires du Nord-Cotentin. pp. 210. 2002.

Halliwell, B.; Gutteridge, J.M.C. Free Radicals in Biology and Medicine. 4th ed. pp. 851. New York: Oxford University Press. 2007.

Hardeland, R.; Coto-Montes, A.; Poeggeler, B. Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. Chronobiology International. 20:921–962. 2003. doi: 10.1081/CBI-120025245.

Helsel, M.E.; Franz, K.J. Pharmacological activity of metal binding agents that alter copper bioavailability. Dalton Transactions. 44:8760-8770. 2015. doi: 10.1039/c5dt00634a.

Hem, J.D. Study and interpretation of the chemical characteristics of natural water. v. 2254. Department of the Interior, US Geological Survey. 1985.

Hirayama, J.; Cho, S.; Sassoni-Corsi, P. Circadian control by the reduction/oxidation pathway: Catalase represses light-dependent clock gene expression in the zebrafish. Proceedings of the National Academy of Sciences of the United States of America, 104:15747–15752. 2007. doi: 10.1073/pnas.0705614104.
Hirayama, J.; Miyamura, N.; Uchida, Y.; Asaoka, Y.; Honda, R.; Sawanobori, K.; Todo, T.; Yamamoto, T.; Sassone-Corsi, P.; Nishina, H. Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish. Cell Cycle. 8:2794-2801. 2009. doi: 10.4161/cc.8.17.9447.

Hoskins, L. J.; Volkoff, H. Daily patterns of mRNA expression of two core circadian regulatory proteins, Clock2 and Per1, and two appetite-regulating peptides, OX and NPY, in goldfish (*Carassius auratus*). Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology. 163:127–136. 2012. doi: 10.1016/j.cbpa.2012.05.197.

Hooven, L. A.; Sherman, K. A.; Butcher, S.; Giebultowicz, J. M. Does the clock make the poison? Circadian variation in response to pesticides. PLoS ONE, 4(7). 2009. doi: 10.1371/journal.pone.0006469.

Hulak, M.; Gazo, I.; Shaliutina, A.; Linhartova, P. In vitro effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 158: 64-71. 2013. doi: 10.1016/j.cbpc.2013.05.002.

Hur, S.P.; Takeuchi, Y.; Itoh, H.; Uchimura, M.; Takahashi, K.; Kang, H. C.; Lee, Y.D.; Kim, S.J.; Takemura, A. Fish sleeping under sandy bottom: Interplay of melatonin and clock genes. General and Comparative Endocrinology. 177:37–45. 2012. doi: 10.1016/j.ygcen.2012.01.007.

Idda, M. L.; Bertolucci, C.; Vallone, D.; Gothilf, Y.; Sánchez-Vázquez, F.J.; Foulkes, N.S. Circadian clocks: lessons from fish. Progress in Brain Research. 199:41–57. 2012. doi: 10.1016/B978-0-444-59427-3.00003-4.

Jaishankar, M.; Tseten, T.; Anbalagan, N.; Mathew, B.B.; Beeregowda, K.N. Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary Toxicology. 7:60–72. 2014. doi: 10.2478/intox-2014-0009.

Jeong, C.B.; Kang, H.M.; Lee, M.C.; Kim, D.H.; Han, J.; Hwang, D.S.; Souissi, S.; Lee, S.; Shin, K.; Park,H.G.; Lee, J.S. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. Scientific reports. 7:41323. 2017. doi: 10.1038/srep41323.

Jiménez-Ortega, V.; Cano-Barquilla, P.; Scacchi, P.; Cardinali, D.P.; Esquifino, A.I. Cadmium-Induced Disruption in 24-h Expression of Clock and Redox Enzyme Genes in Rat Medial Basal Hypothalamus: Prevention by Melatonin. Frontiers in Neurology. 2:13. 2011. doi:10.3389/fneur.2011.00013.

Jin, Y.; Zhang, X.; Shu, L.; Chen, L.; Sun, L.; Qian, H.; Liu, W.; Fu, Z. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). Chemosphere. 78:846–852. 2010. doi: 10.1016/j.chemosphere.2009.11.044.

Karan, V.; Vitorovic, S.; Tutundzic, V.; Poleksic, V. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. Ecotoxicology and Environmental Safety. 40:49–55. 1998. doi: 10.1006/eesa.1998.1641.

Kondratov, R.V. A role of the circadian system and circadian proteins in aging. Ageing Research Reviews. 6:12–27. 2007. doi:10.1016/j.arr.2007.02.003.

Kono, Y. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Archives of Biochemistry and Biophysics. 186:189-195. 1978. doi:10.1016/0003-9861(78)90479-4.

Krishnan, N.; Davis, A.J.; Giebultowicz, J.M. Circadian regulation of response to oxidative stress in *Drosophila melanogaster*. Biochemical and Biophysical Research Communications. 374:299–303. 2008. doi: 10.1016/j.bbrc.2008.07.011.

Kulczykowska, E.; Popek, W.; Kapoor, B.G. Biological clock in fish. 1st edition, CRC Press.pp.280. 2010.

Lerebours, A.; Gonzalez, P.; Adam, C.; Camilleri, V.; Bourdineaud, J.P.; Garnier-Laplace, J. Comparative analysis of gene expression in brain, liver, skeletal muscles, and gills of zebrafish (*Danio rerio*) exposed to environmentally relevant waterborne uranium concentrations. Environmental Toxicology and Chemistry. 28:1271-1278. 2009. doi: 10.1897/08-357.1.

Livingstone, D.R. Contaminant-stimulates reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin.42:656-666. 2001. doi: 10.1016/S0025-326X(01)00060-1.

López-Olmeda, J.F.; Egea-Álvarez, M. Sánchez-Vázquez, F. J. Glucose tolerance in fish: Is the daily feeding time important? Physiology and Behavior. 96:631–636. 2009. doi: 10.1016/j.physbeh.2008.12.015.

Macfarlane, R.D.; Bullock, G.L.; Mclaughlin, J.J.A. Effects of five metals on susceptibility of striped bass to Flexibacter columnaris. Trans. A Fish. Soc. 115: 227–231. 1986.

Manfredi, G.; Yang, L.; Gajewski, C.D.; Mattiazzi, M. Measurements of ATP in mammalian cells. Methods. 26:317-326. 2002. doi: 10.1016/S1046-2023(02)00037-3.

Martin, V.; Sainz, R.M.; Mayo, J.C.; Antolin, I.; Herrera, F.; Rodriguez, C. Daily rhythm of gene expression in rat superoxide dismutases. Endocrine Research. 29:83–95. 2003. doi: 10.1081/ERC-120018679.

Mattie, M.D.; McElwee, M.K.; Freedman, J.H. Mechanism of copper-activated transcription: activation of AP-1, and the JNK/SAPK and p38 signal transduction pathways. Journal of Molecular Biology. 383:1008-1018. 2008. doi: 10.1016/j.jmb.2008.08.080.

McElwee, M.K.; Song, M.O.; Freedman, J.H. Copper activation of NF-κB signaling in HepG2 cells. Journal of Molecular Biology. 393:1013-1021. 2009. doi: 10.1016/j.jmb.2009.08.077.

Mela, M.; Guiloski, I.C.; Doria, H.B.; Rabitto, I.S.; Silva, C.A.; Maraschi, A.C.; Prodocimo, V.; Freire, C.A.; Randi, M.A.F.; Oliveira Ribeiro, C.A.; Assis, H.C.S. Risks of waterborne copper exposure to a cultivated freshwater Neotropical catfish (*Rhamdia quelen*). Ecotoxicology and Environmental Safety. 88:108–16. 2013. doi: 10.1016/j.ecoenv.2012.11.002.

Moore, H.; Whitmore, D. Circadian rhythmicity and light sensitivity of the zebrafish brain. PloS One, 9(1), e86176. 2014. doi: 10.1371/journal.pone.0086176.

Mracek, P.; Santoriello, C.; Idda, M.L.; Pagano, C.; Ben-Moshe, Z.; Gothilf, Y.; Vallone, D.; Foulkes, N. S. (2012). Regulation of per and cry Genes Reveals a Central Role for the D-Box Enhancer in Light-Dependent Gene Expression. PLoS ONE. 7(12). 2012. doi: 10.1371/journal.pone.0051278.

Mracek, P.; Pagano, C.; Frohlich, N.; Idda, M.L.; Cuesta, I.H.; Lopez-Olmeda, J.F.; Sanchez-Vazquez, F.J.; Vallone, D.; Foulkes, N.S. ERK Signaling Regulates Light-Induced Gene Expression via D-Box Enhancers in a Differential, Wavelength-Dependent Manner. PLoS ONE. 8:1–10. 2013. doi: 10.1371/journal.pone.0067858.

Mukaka, M.M. Statistics corner: A guide to appropriate use of correlation coefficient in medical research. Malawi Medical Journal. 24:69–71. 2012.

Nader, N.; Chrousos, G.P.; Kino, T. Interactions of the circadian CLOCK system and the HPA axis. Trends in Endocrinology & Metabolism. 21:277-286. 2010. doi: 10.1016/j.tem.2009.12.011.

Navarro, R.F. A evolução dos materiais. Parte 1: da Pré-história ao início da Era Moderna. REMAP. 1(1): 01-1. 2006.

Nordberg, J.; Arnér, E.S.J. Reactive oxygen species antioxidants and the mammalian thioredoxin system. Free Radical Biology and Medicine. 31:1287-1311. 2001. doi: 10.1016/S0891-5849(01)00724-9.

O'Neill, J.S.; Feeney, K.A. Circadian Redox and Metabolic Oscillations in Mammalian Systems. Antioxidants & Redox Signaling, 20:2966–2981. 2014. doi: 10.1089/ars.2013.5582.

Pando, M.P.; Pinchak, B.; Cermakian, N.; Sassone-Corsi, P. A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. Proceedings of the National Academy of Sciences of the United States of America. 98:10178–10183. 2001. doi: 10.1073/pnas.181228598.

Pando, M.P.; Sassone-Corsi, P. Unraveling the mechanisms of the vertebrate circadian clock: zebrafish may light the way. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology. 24:419–426. 2002. doi: 10.1002/bies.10091.

Paquin, P.R.; Gorsuch, J.W.; Apte, S.; Batley, G.E.; Bowles, K.C.; Campbell, P.G.C.; Delos, C.G.; Di Toro, D.M.; Dwyer, R.L.; Galvez, F.; Genzemer, R. W.; Goss, G.G.; Hogstrand, C.; Janssen, C.R.; Pavanelli, G.C.; Eiras, J.C.; Takemoto, R.M. Doenças de peixes: profilaxia diagnóstico e tratamento, 2 ed. Eduem, Maringá. 2002.

Patel, S.; Velingkaar, N.S.; Kondratov, R.V. Transcriptional control of antioxidant defense by the circadian clock. Antioxidants & Redox Signaling. 20:2997–3006. 2014. doi: 10.1089/ars.2013.5671.

Peyric, E.; Moore, H.A.; Whitmore, D. Circadian clock regulation of the cell cycle in the zebrafish intestine. PloS One. 8(8):e73209. 2013. doi: 10.1371/journal.pone.0073209.
Rhee, J.S.; Kim, B.M.; Lee, B.Y.; Hwang, U.K.; Lee, Y.S.; Lee, J.S. Cloning of circadian rhythmic pathway genes and perturbation of oscillation patterns in endocrine disrupting chemicals (EDCs)-exposed mangrove killifish *Kryptolebias marmoratus*. Comparative Biochemistry and Physiology. Toxicology & Pharmacology. 164:11–20. 2014. doi: 10.1016/j.cbpc.2014.04.001.

Ramos, B.C.; Moraes, M.N.C.; Poletini, M.O.; Lima, L.H.; Castrucci, A.M.L. From blue light to clock genes in zebrafish ZEM-2S cells. PLoS One. 9:e106252. 2014. doi: 10.1371/journal.pone.0106252.

Robins, R.G.; Berg, R.B.; Dysinger, D.K.; Duaime, T.E.; Metesh, J.J.; Diebold, F.E.; Twidwell L.; Mitman, G.; Chatham, W.; Huang, H.; Young, C.A. Chemical, physical and biological interaction at the Berkeley Pit, Butte, Montana. Tailings and Mine Waste. v.97, pp. 529-541. Rotterdam, The Netherlands: Bakeman. 1997.

Rutter, J.; Reick, M.; McKnight, S.L. Metabolism and the control of circadian rhythms. Annual Review of Biochemistry, 71:307–331. 2002. doi: 10.1146/annurev.biochem.71.090501.142857.

Sani, M.; Sebai, H.; Gadacha, W.; Boughattas, N.; Reinberg, A.; Mossadock, B.A. Catalase activity and rhythmic patterns in mouse brain, kidney and liver. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 145:331-337.2006. doi: 10.1016/j.cbpb.2006.08.005.

Shi, X.; Zhou, B. The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. Toxicological Sciences, 115:391-400. 2010. doi: 10.1093/toxsci/kfq066.

Silva Acosta, D.; Danielle, N.M.; Altenhofen, S.; Luzardo, M.D.; Costa, P.G.; Bianchini, A.; Bonan, C.D.; Silva, R.S.; Dafre, A.L. Copper at low levels impairs memory of adult zebrafish (*Danio rerio*) and affects swimming performance of larvae. Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology. 185:122–130. 2016. doi: 10.1016/j.cbpc.2016.03.008.

Simonato, J. D.; Mela, M.; Doria, H.B.; Guiloski, I.C.; Randi, M.A.F.; Carvalho, P.S.; Meletti, P.C.; Silva de Assis, H.C.; Bianchini, A.; Martinez, C.B.R. Biomarkers of waterborne copper exposure in the Neotropical fish *Prochilodus lineatus*. Aquatic Toxicology. 170:31-41. 2015. doi: 10.1016/j.aquatox.2015.11.012.

Sloman K.A.; Scott, G.R.; Diao, Z.; Rouleau, C.; Wood, C.M.; Mcdonald, D.G. Cadmium affects the social behaviour of rainbow trout, *Oncorhynchs mykiss*. Aquatic Toxicology. 65:171-185. 2003. doi: 10.1016/S0166-445X(03)00122-X.

Song, M.O.; Li, J.; Freedman, J.H. Physiological and toxicological transcriptome changes in HepG2 cells exposed to copper. Physiological Genomics. 38:386-401. 2009. doi: 10.1152/physiolgenomics.00083.2009.

Straus, D.L.; Tucker, C.S. Acute toxicity of copper sulfate and chelated copper to channel catfish *Ictalurus puntatus*. Journal of the World Aquaculture Society. 24: 390-395. 1993. doi:10.1111/j.1749-7345.1993.tb00170.x.

Subramanian, P.; Dakshayani, K.B.; Pandi-Perumal, S.R.; Trakht, I.; Cardinali, D.P. 24-hour rhythms in oxidative stress during hepatocarcinogenesis in rats: effect of melatonin or alpha-ketoglutarate. Redox Report: Communications in Free Radical Research. 13:78–86. 2008. doi: 10.1179/135100008X259178.

Tamai, T.K.; Young, L.C.; Whitmore, D. Light signaling to the zebrafish circadian clock by Cryptochrome 1a. Proceedings of the National Academy of Sciences of the United States of America. 104:14712–14717. 2007. doi: 10.1073/pnas.0704588104.

Tseng, H.L.; Li, C.J.; Huang, L.H.; Chen, C.Y.; Tsai, C.H.; Lin, C.N.; Hsu, H.Y. Quercetin 3-O-methyl ether protects FL83B cells from copper induced oxidative stress through the PI3K/Akt and MAPK/Erk pathway. Toxicology and Applied Pharmacology. 264:104-113. 2012. doi: 10.1016/j.taap.2012.07.022.

Turski, M.L.; Brady, D.C.; Kim, H.J.; Kim, B.E.; Nose, Y.; Counter, C.M.; Thiele, D.J. A novel role for copper in Ras/mitogen-activated protein kinase signaling. Molecular and Cellular Biology, 32:1284-1295. 2012. doi: 10.1128/MCB.05722-11.

US EPA- United States Environmental Protection Agency. Ambiental water quality criteria for copper. Washington, DC. 1984.

Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology. 13:57–149. 2003. doi: 10.1016/S1382-6689(02)00126-6.

Valko, M.; Morris, H.; Cronin, M.T.D. Metals, Toxicity and oxidative stress. Current Medicinal Chemistry. 12:1161-1208. 2005. doi: 10.2174/0929867053764635.

Vallone, D.; Gondi, S.B.; Whitmore, D.; Foulkes, N.S. E-box function in a period gene repressed by light. Proceedings of the National Academy of Sciences of the United States of America. 101: 4106–4111. 2004. doi: 10.1073/pnas.0305436101.

Varo, I.; Nunes, B.; Amat, F.; Torreblanca, A.; Guilhermino, L.; Navarro, J.C. Effect of sublethal concentrations of copper sulphate on seabream *Sparus aurata* fingerlings. Aquat. Living Resour. 20:263-270. 2007. doi: 10.1051/alr:2007039.

Vatine, G.; Vallone, D.; Gothilf, Y.; Foulkes, N. S. It's time to swim! Zebrafish and the circadian clock. FEBS Letters. 585:1485–94. 2011. doi: 10.1016/j.febslet.2011.04.007.

Velardea, E.; Haqueb, A.; Iuvoneb, P.M.; Azpeleta, C.; Alonso-Gómez, A.L.; Delgado, M.J.D. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of Period and Cryptochrome transcripts in retina, liver, and gut. Journal of Biological Rhythms. 24: 104–113. 2009.doi: 10.1177/0748730408329901 24(2):104–113. 2010.

Vieira, L.R.; Gravato, C.; Soares, M.V.M.; Morgado, F.; Guilhermino, L. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. Chemosphere. 76:1416–27. 2009. doi: 10.1016/j.chemosphere.2009.06.005.

Volpato, G. L.; Trajano, E. Biological rhythms. In: Fish Physiology: The Physiology of Tropical Fishes: The Physiology of Tropical Fishes. p.21-101. 2005.

Wang, H. Comparative analysis of teleost fish genomes reveals preservation of different ancient clock duplicates in different fishes. Marine Genomics. 1:69–78. 2008. doi: 10.1016/j.margen.2008.06.003.

Wang, X.J.; Hayes, J.D.; Higgins, L.G.; Wolf, C.R.; Dinkova-Kostova, A.T. Activation of the NRF2 signaling pathway by copper-mediated redox cycling of para-and ortho-hydroquinones. Chemistry & Biology. 17:75-85. 2010. doi: 10.1016/j.chembiol.2009.12.013.

Weger, M.; Weger, B.D.; Diotel, N.; Rastegar, S.; Hirota, T.; Kay, S.A.; Strahle, U.; Dickmeis, T. Real-time in vivo monitoring of circadian E-box enhancer activity: a robust and sensitive zebrafish reporter line for developmental, chemical and neural biology of the circadian clock. Developmental Biology. 380:259–273. 2013. doi: 10.1016/j.ydbio.2013.04.035.

Wendelaar Bonga, S. E. The Stress Response in Fish. Physiological Reviews. 77: 591-620. 1997.

Whitmore, D.; Foulkes, N.S.; Sassone-Corsi, P. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature. 404:87–91. 2000. doi: 10.1038/35003589.

Wu, L.; Reddy, A.B. Rethinking the clockwork: redox cycles and non-transcriptional control of circadian rhythms. Biochemical Society Transactions, 42:1–10. 2014. doi:10.1042/BST20130169.

Xu, Y.Q.; Zhang, D.; Jin, T.; Cai, D.J.; Wu, Q.; Lu, Y.; Liu, J.; Klaassen, C.D. Diurnal Variation of Hepatic Antioxidant Gene Expression in Mice. PLoS ONE. 7(8):e44237. 2012. doi: 0.1371/journal.pone.0044237.

Zelinski, E. L.; Deibel, S. H.; Mcdonald, R.J. The trouble with circadian clock dysfunction: multiple deleterious effects on the brain and body. Neuroscience and Behavioral Reviews, 40:80–101. 2014. doi: 10.1016/j.neubiorev.2014.01.007.

Ziv, L.; Levkovitz, S.; Toyama, R.; Falcon, J.; Gothilf, Y. Functional development of the zebrafish pineal gland: Light-induced expression of period2 is required for onset of the circadian clock. Journal of Neuroendocrinology. 17:314–320. 2005. doi: 10.1111/j.1365-2826.2005.01315.x.

Zhou, J.; Zhu, X.S.; Cai, Z.H. The impacts of bisphenol A (BPA) on abalone (*Haliotis diversicolor supertexta*) embryonic development. Chemosphere. 82: 443-450. 2011. doi: 10.1016/j.chemosphere.2010.09.056.

APPENDIX

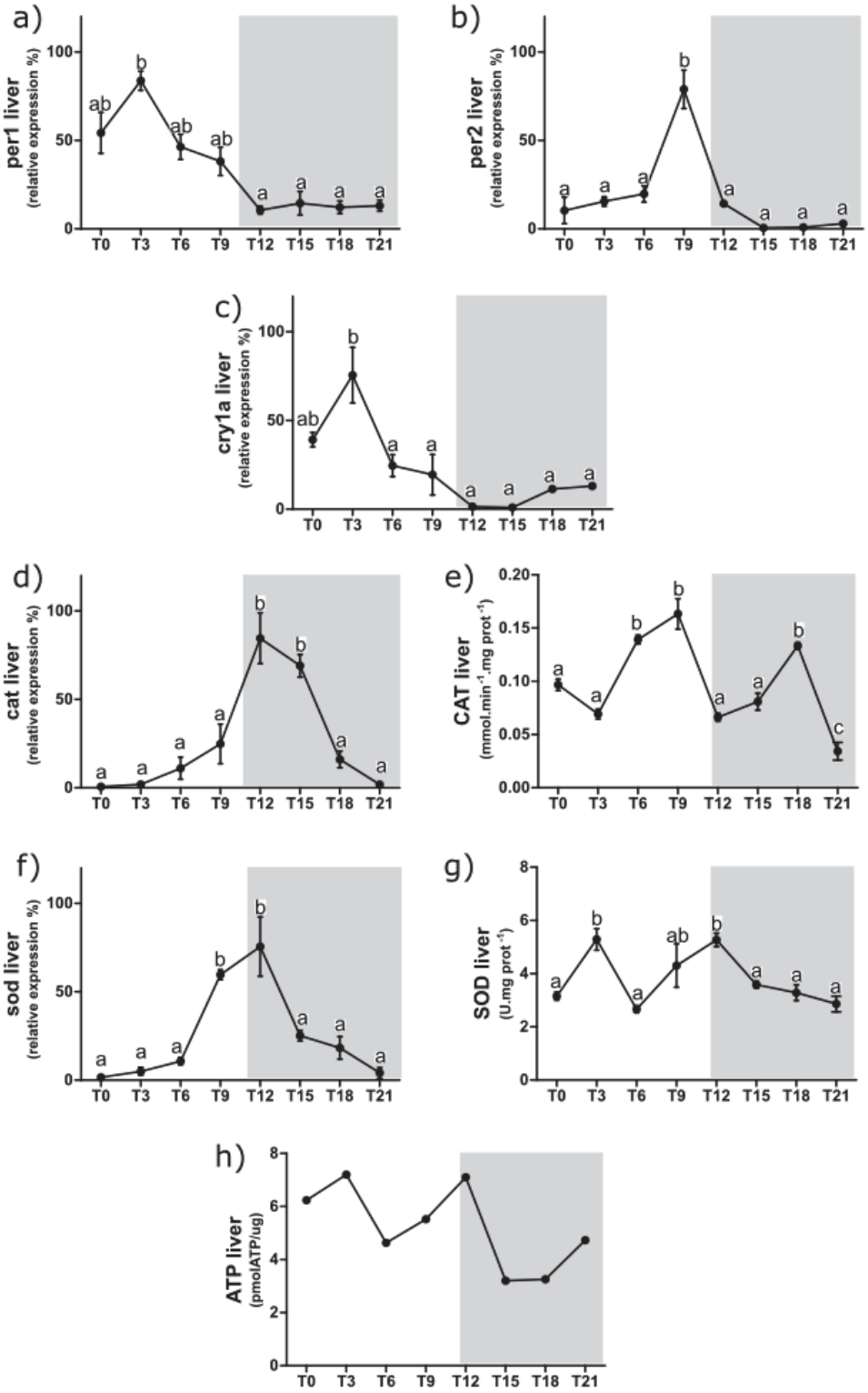


Fig. 1: Biological responses in *Danio rerio* liver under control conditions. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); h) intracellular ATP concentration (pmolATP/ug). Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15 for all parameters evaluated; except ATP concentration n=5).

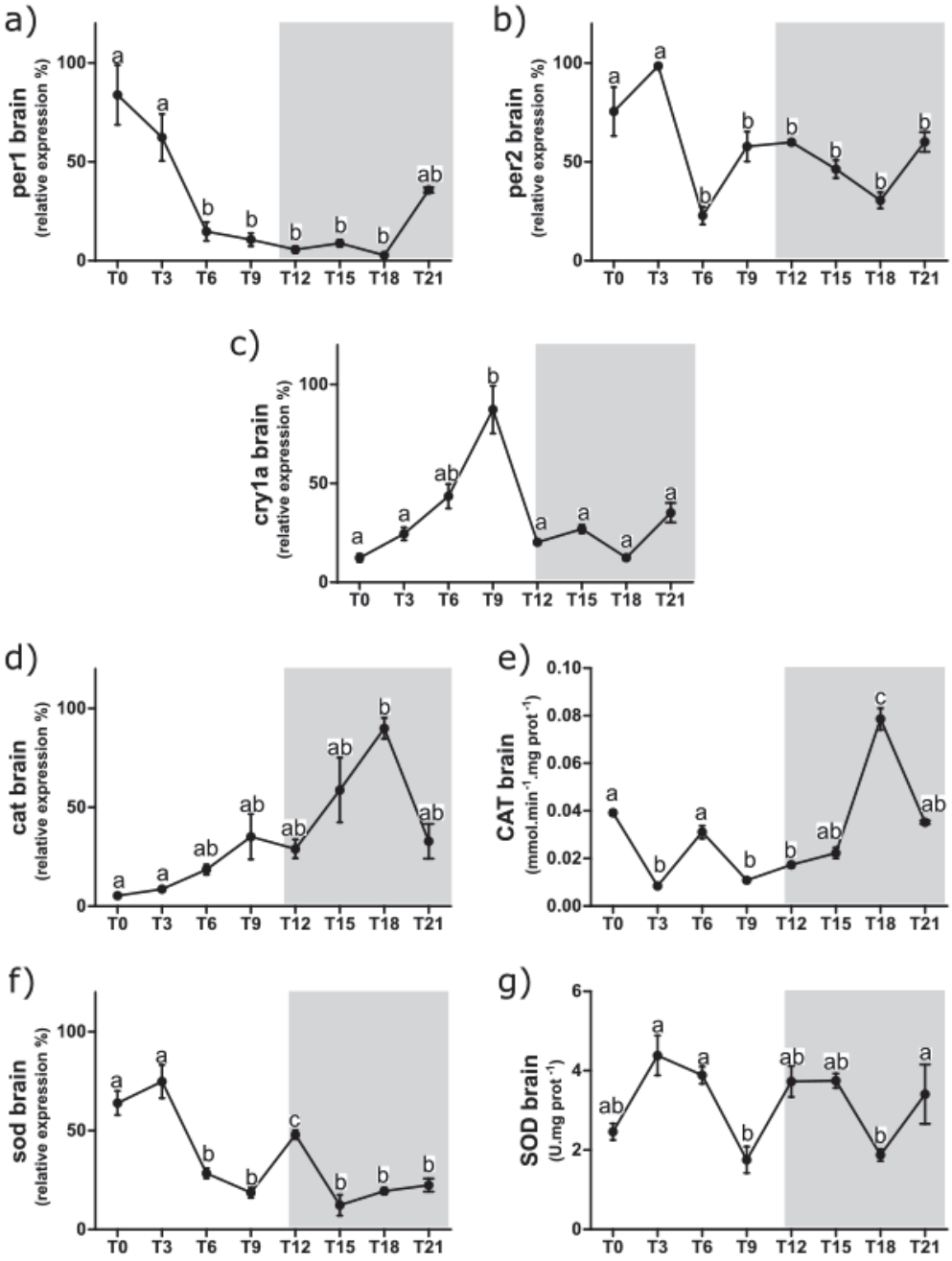


Fig. 2: Biological responses in *Danio rerio* brain under control conditions. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15).

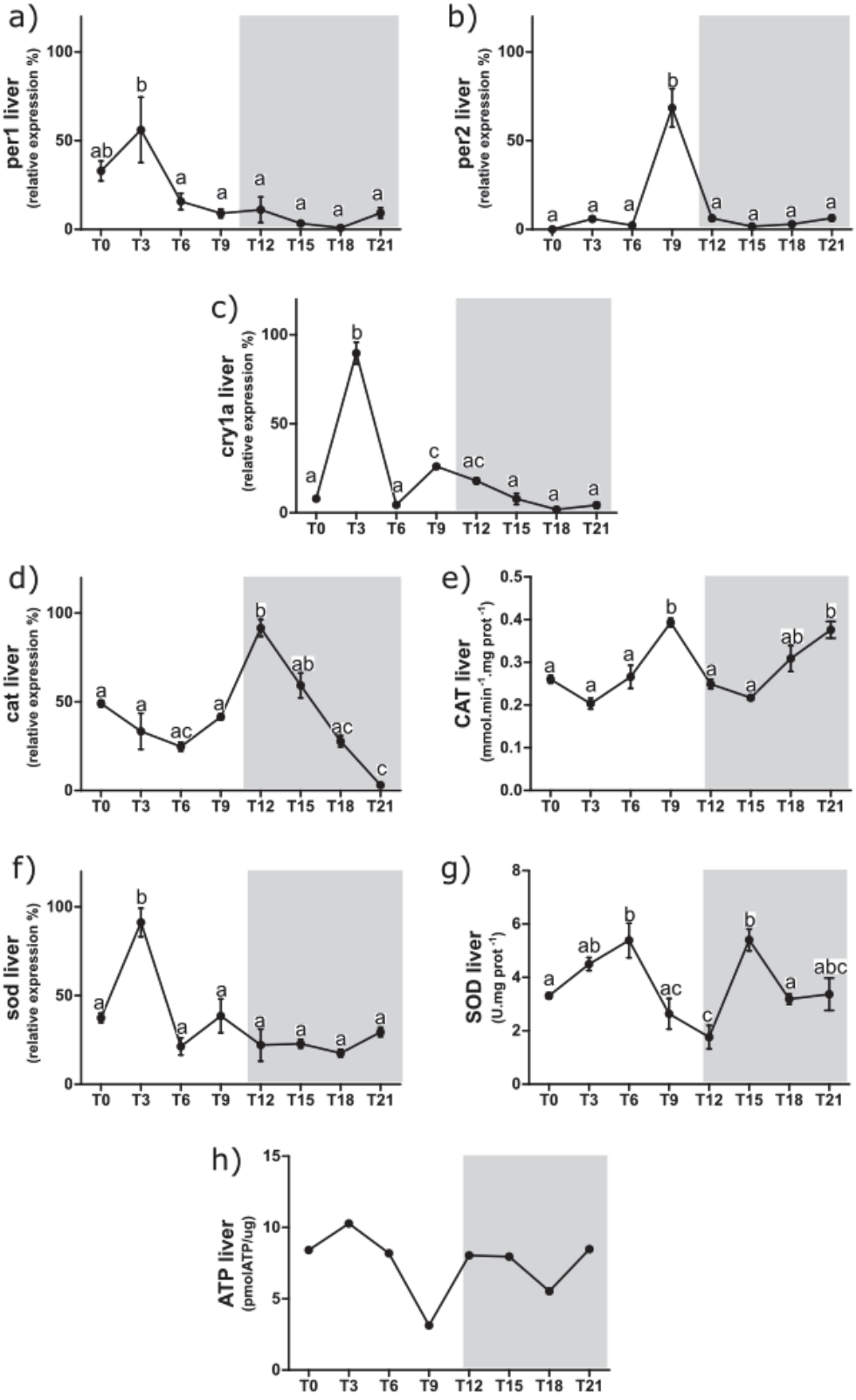


Fig. 3: Biological responses in liver from *Danio rerio* exposed to the lowest $CuSO_4$ concentration - 5µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); h) intracellular ATP concentration (pmolATP/ug). Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15 for all parameters evaluated; except ATP concentration n=5).

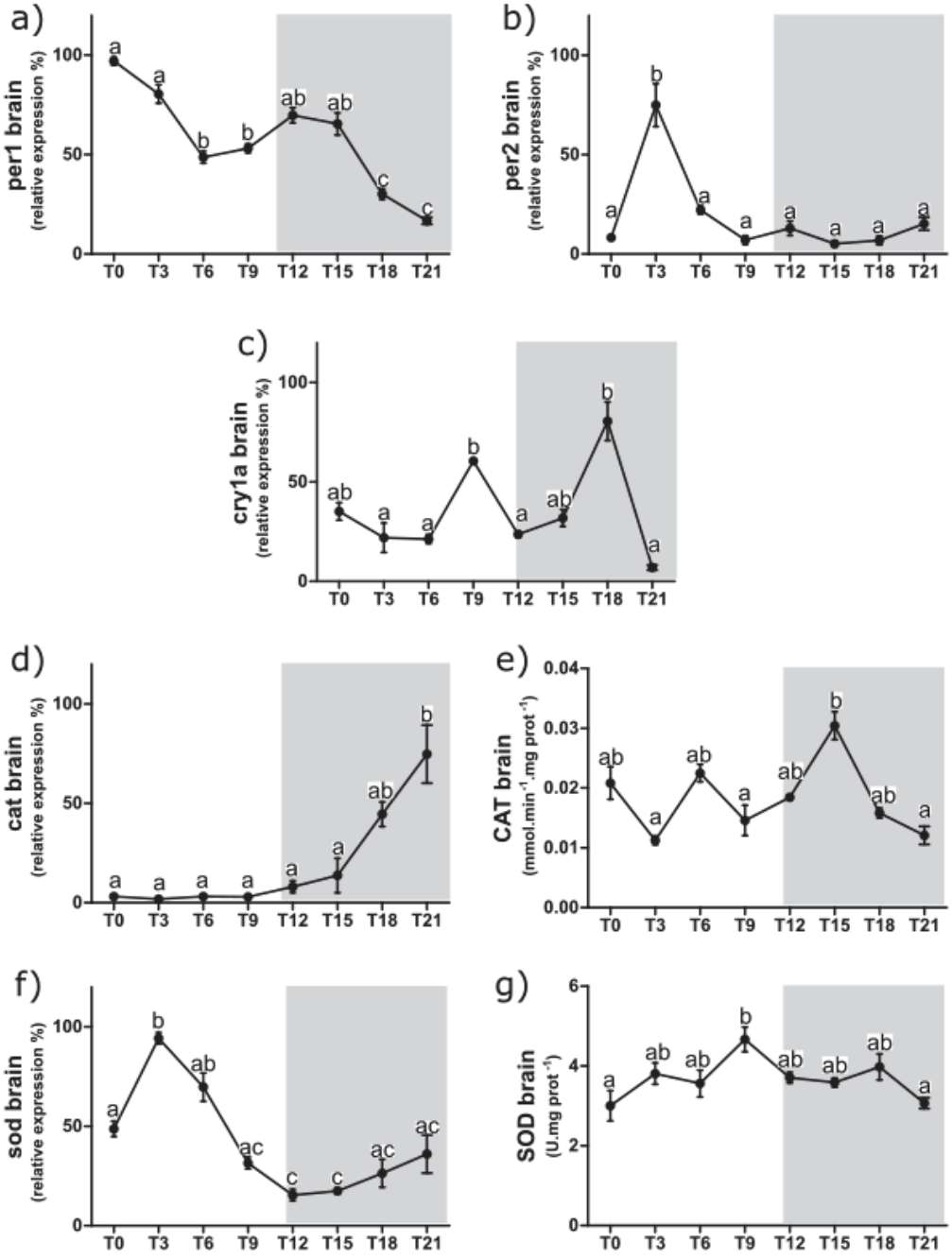


Fig. 4: Biological responses in brain from *Danio rerio* exposed to the lowest $CuSO_4$ concentration - 5µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15).

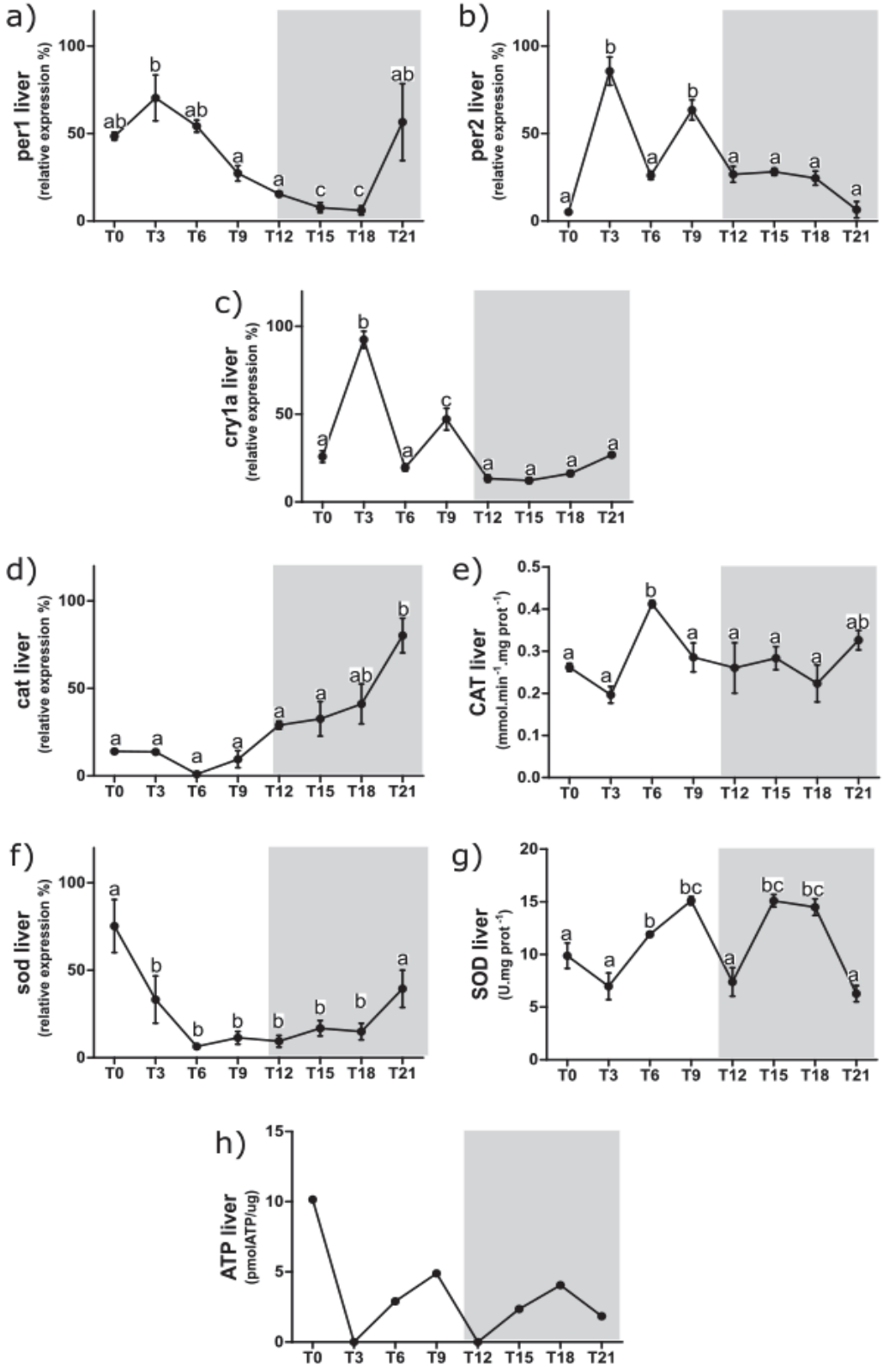


Fig. 5: Biological responses in liver from *Danio rerio* exposed to the intermediate $CuSO_4$ concentration - 25µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); h) intracellular ATP concentration (pmolATP/ug). Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15 for all parameters evaluated; except ATP concentration n=5).

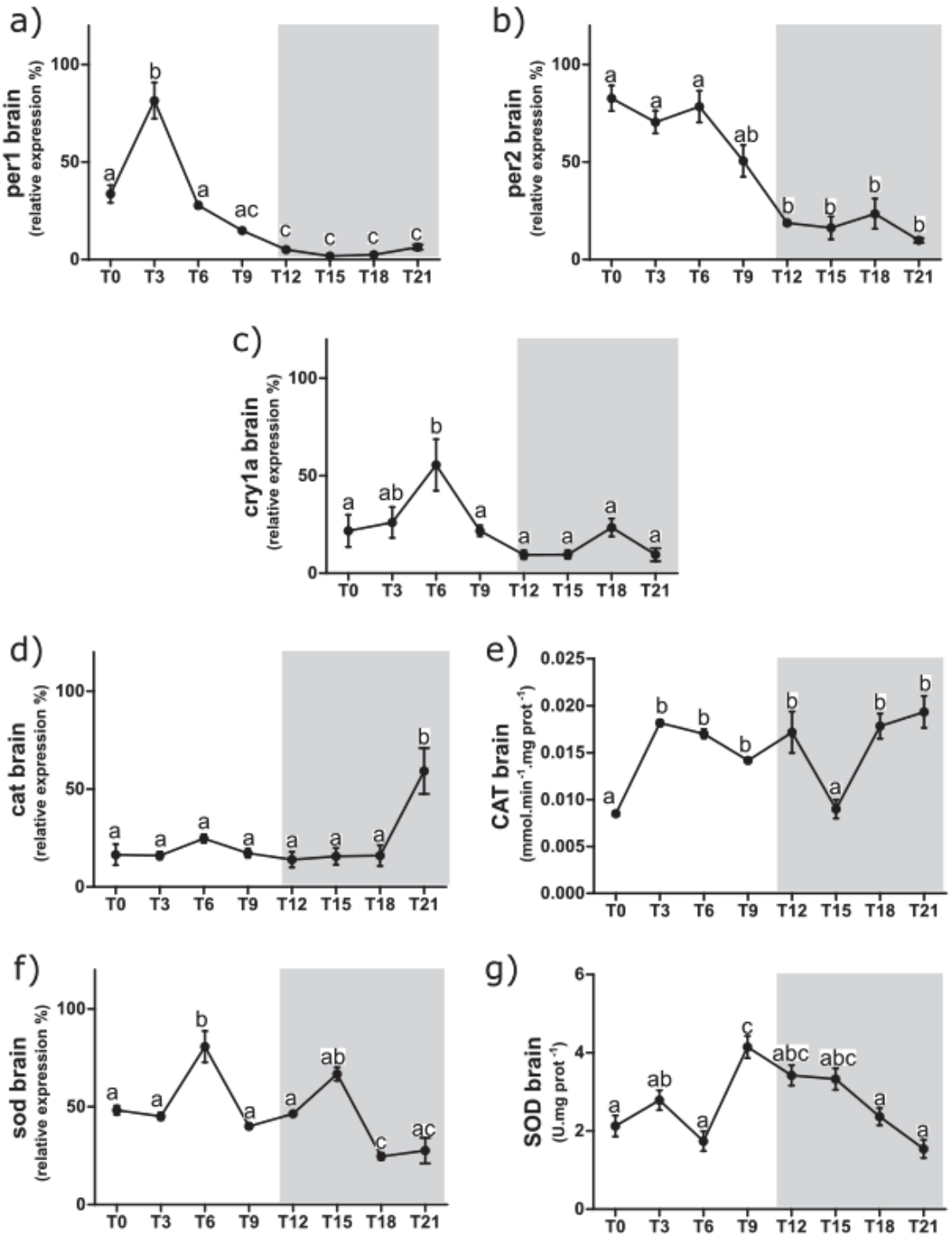


Fig. 6: Biological responses in brain from *Danio rerio* exposed to the intermediate $CuSO_4$ concentration - 25µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15).

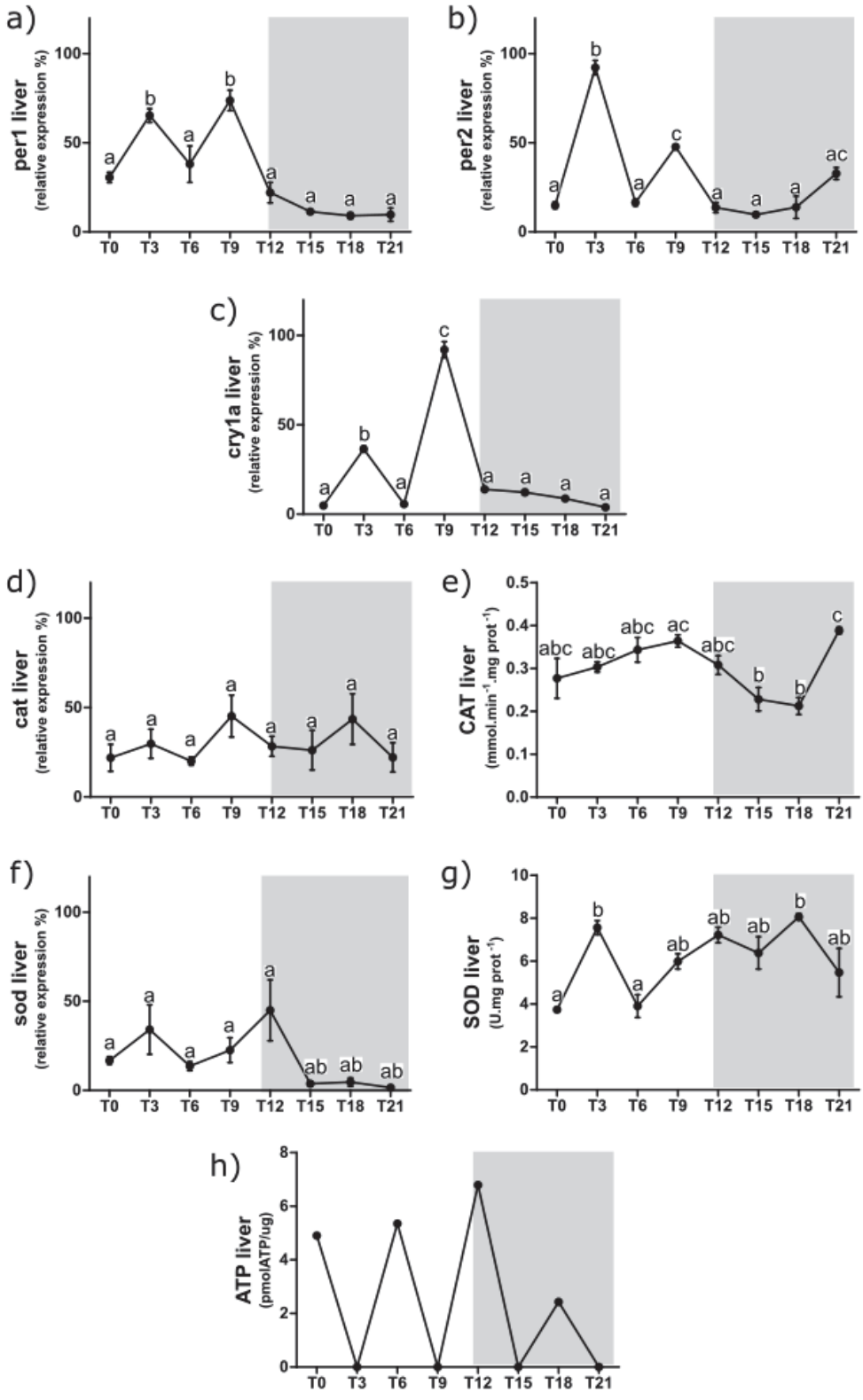


Fig. 7: Biological responses in liver from *Danio rerio* exposed to the highest $CuSO_4$ concentration - 45µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); h) intracellular ATP concentration (pmolATP/ug). Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15 for all parameters evaluated; except ATP concentration n=5).

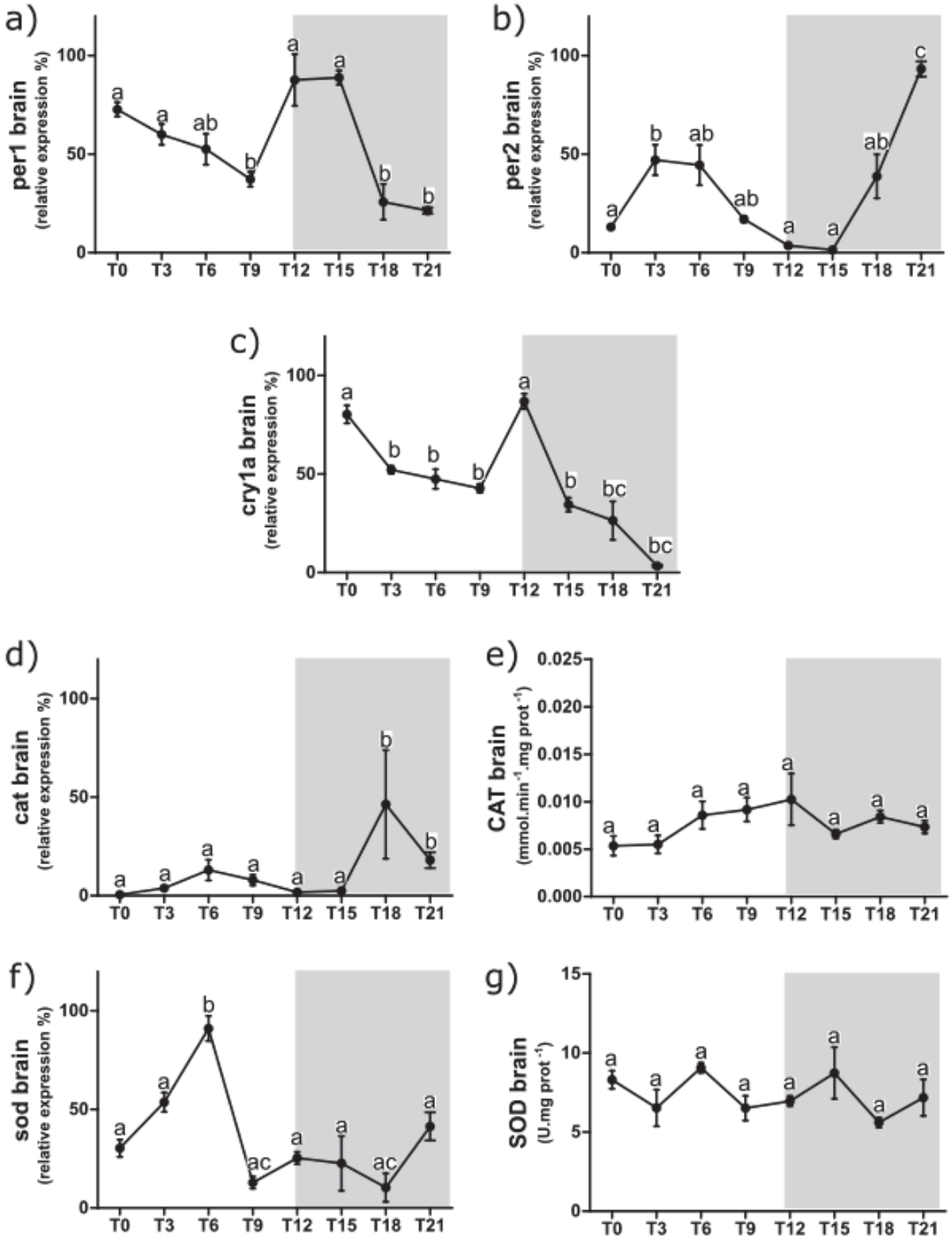


Fig. 8: Biological responses in brain from *Danio rerio* exposed to the highest $CuSO_4$ concentration - 25µg/L. The grey area represents the dark period. a) endogenous per1 relative expression (%); b) endogenous per2 relative expression (%); c) endogenous cry1a relative expression (%); d) endogenous cat relative expression (%); e) specific CAT activity (mmol of degraded H_2O_2/min/mg protein); f) endogenous relative SOD expression (%); g) specific SOD activity (U/hour/mg protein); Different letters denote significant differences ($p<0.05$). Data are expressed as means ± standard error of the means (n=15).

6. CAPÍTULO 2

The Cross-Talk Between Light, SOD and the Circadian Clock in the Zebrafish (*Danio rerio*) and its Deregulation by Copper Exposure

Artigo submetido à ser submetido

Halina Binde Doria[1], Daniela Vallone[2], Marco Antonio Ferreira Randi[1], Nicholas Simon Foulkes[2]

[1]Federal University of Paraná (UFPR), Department of Cellular Biology, School of Biological Sciences, Address: Caixa Postal 19.031, Curitiba, Paraná, Brazil, Postal Code: 81531-990.

[2] Karlsruher Institute für Technologie (KIT), Institute of Toxicology and Genetics, Address: 76344, Eggenstein-Leopoldshafen, Baden-Württemberg, Germany.

Declarations of interest: none

Abstract

Copper (Cu) is essential to life, but at high concentrations it can induce the generation of reactive oxygen species (ROS) and consequently, induce the antioxidant defense. Both Cu and light can generate ROS and were demonstrated to activate the MAPK pathway, that is implicated on the transcription of the light regulated genes and the antioxidant defense. That way, a close connection between the circadian clock and antioxidant defenses exists, but little is known about the molecular mechanisms underlying this cross-talk. The present study aimed to address the interaction between the circadian and antioxidant systems, by studying the transcriptional control of the cytosolic superoxide dismutase (SOD1), the first line of defense against ROS, and whether the response to acute oxidative stress caused by $CuSO_4$ exposure can modulate or be modulated by the circadian system. The results presented strongly support a relationship between $CuSO_4$ acute exposure, activation of antioxidant defense response and impaired cyclical expression, dampened amplitude, and altered phase of genes involved in the circadian clock in PAC-2 cells. Further, it was also demonstrated that zebrafish expression of SOD1 can be regulate either by ARE ligands and PAR/bZIP transcription factors, being SOD1 transcription activated by oxidative stress and coupled with the circadian clock.

Key words: Superoxide dismutase, Circadian clock, Copper sulfate, *Danio rerio*

1. Introduction

Copper (Cu) is a micronutrient essential to life. Its electron structure allows to interreact with spin-restricted dioxygen, permitting Cu to act as a co-factor for numerous enzymes and be involved in fundamental redox reactions, cellular respiration and free radical defense (Harris and Gitlin, 1996). However, the same properties that make Cu biologically valuable can also make it potentially toxic (Santos et al., 2009; Zavitsanos et al., 2011; Simonato et al., 2015). Thus, when in excess, Cu can induce stress either by redox cycling that results in the generation of reactive oxygen species (ROS) and other damaging intermediates (Pham et al., 2013) or by depletion of glutathione which allow that endogenous levels of ROS become cytotoxic (Mattie and Freedman, 2004; Valko et al., 2005; Helsel & Franz, 2015).

It has been demonstrated that ROS can interfere with cellular survival, growth and proliferation, apoptosis control and circadian clock regulation (Dröge, 2002; Li et al., 2004; Hirayama et al., 2007). Although ROS are constantly produced by the normal cell metabolism, depending if its concentration is maintained below a toxic threshold, it holds both cell-activating and cell-impairing properties (Nordberg & Arnér, 2001; Dröge, 2002; Wang et al., 2010; Tseng et al., 2012). Oxidative stress and cellular damage occurs when there is an imbalance in intracellular ROS homeostasis, whether through an increase in ROS levels or a decrease in the cellular antioxidant capacity (Wendelaar Bonga, 1997; Livingstone, 2001; Patel et al., 2014).

Consequently, to maintain intracellular ROS homeostasis cells hold an intricate detoxification system mediated by nonenzymatic molecules and antioxidant enzymes that specifically scavenge different kinds of ROS (Hardeland et al., 2003; Volpato & Trajano, 2005; Storz, 2011; Patel et al., 2014). In the first defense line, superoxide dismutase (SOD) is responsible for the dismutation of $\bullet O_2$ anions to H_2O_2 and, for that reason, is an antioxidant enzyme that occurs in virtually all oxygen respiring organisms (Cheng et al., 2006; Craig et al., 2007). Three different isoforms of SOD were identified and they exist within different cell compartments: Mn-SOD or SOD2 is found in the mitochondrial matrix and the other two isoforms have Cu and Zn in their catalytic center, SOD3 or EC-SOD is located extracellularly and SOD1 or Cu/Zn-SOD is mainly found in the cytosol, but is also present in the nucleus, the lysosomes and the mitochondria (Zelko et al., 2002; Storz, 2011; Milani et al., 2011). Moreover, SOD1 activity and gene expression are identified as indicators of the antioxidant capacity of an organism (Yin et al., 2018).

Interestingly, recent studies pointed to a close connection between the circadian clock, redox signaling and antioxidant defenses (Rutter et al., 2002; Hardeland et al., 2003; Krishnan et al., 2008; Jimenéz-Ortega et al., 2011; O´Neill & Feeny, 2014; Wu & Reddy, 2014). Also, disorders of rhythmicity and in redox homeostasis have implications in the so-called modern epidemics, such as diabetes, cancer, depression and cardiovascular disease (Milani et al., 2011; Zelinski et al., 2014). Light and Cu can generate ROS and were demonstrated to activate the MAPK pathway, that is implicated on the transcription of the light regulated genes and the antioxidant defense (Ostrakhovitch et al., 2002; Hirayama et al., 2007; Mattie et al., 2008; Song & Freedman, 2009; McElwee et al., 2009; Wang et al., 2010; Tseng et al., 2012; Turski et al., 2012). Although, the cross-talk between antioxidant defense and the circadian clock seems evident, it may occur through several different transcriptional controls. For example, it can be under direct regulation by the circadian clock transcriptional factors (BMAL1/CLOCK, RORs and Rev-Erbs) or clock-controlled transcriptional factors (PARbZip proteins) or through clock-dependent control of chromatin structure. Nevertheless, molecular mechanisms and key regulators of those processes are yet unstudied (Patel et al., 2014).

The present study aimed to address the interaction between the circadian and antioxidant systems, by studying more closely the promoter of SOD and whether the response to acute oxidative stress caused by $CuSO_4$ exposure can modulate or be modulated by the circadian system.

2. *Material and methods*

2.1.In vitro Fish Cell Cultures

PAC-2 cell line that is derived from zebrafish (Lin et al., 1994) and a stably transfected zebrafish PAC-2 cell line that contain clock and light regulated luciferase reporter per1b-Luc (DAP49) were employed. Both cell lines were established and cultured as described elsewhere (Vallone et al., 2004; Vallone et al., 2007). For incubation under the different lighting regimes performed in the study, cells were maintained under constant temperature of 26ºC in darkrooms or light-sealed incubators and were illuminated with a tungsten light source (20 $\mu W/cm^2$).

2.2.$CuSO_4$ and H_2O_2 Exposure

$CuSO_4$ pentahydrate (MM 249.68 g/mol) was purchased from MERK. Initially, a stock solution of 1M was always prepared fresh before the exposure with autoclaved water and

filtered in 0.22 μm membrane. The stock solution was diluted in L-15 medium (Leibovitz) according to the final copper concentration desired. A stock solution of 30% v/v of H_2O_2 was diluted in L-15 medium (Leibovitz) according to the final H_2O_2 concentration desired. An appropriate control group was kept in parallel and received the same amount of vehicle (water).

2.3.MTT assay (Cell viability and Metabolism)

Cells were plated at $3x10^4$ density in a 96-well plate, treated with different $CuSO_4$ concentrations, as previously described, and incubated for 24 or 48 hours at 26ºC in constant darkness before the assay. Cell viability and metabolism was determined after incubation of cells with 0.5 mg/L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in L-15 medium (Leibovitz) lacking phenol red for 3 hours. After, the medium was removed and DMSO was employed as a solvent. The absorbance was measured at 550 nm by a spectrophotometer.

2.4.Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted based on the TRIzol® method (Chomczynski, 1993). Total RNA was reverse-transcribed into cDNA by using Superscript III Reverse Transcriptase (Invitrogen) with a mixture of oligo dT and random primers. Quantitative RT-PCR analysis was performed using a StepOnePlus Real-Time RT-PCR System (Applied Biosystems) and SYBR Green I fluorescent dye (Qiagen). Primers were purchased from Sigma-Aldrich and are presented in Table 1. For quantification, the ΔCt method was used and β-actin was chosen as the endogenous control.

TABLE 1: Primers used in the qRT-PCR

	Forward	**Reverse**
sod1	ctggccttactccaggaaaac	tgacagagtcagcattgcatc
cry1a	ggctccacgacaatccttca	tggggaagacatcggtaggt
per2	ccgcaaagtttccttcgtca	cattactgcccagactccca
per1	gagagttcattctggatacga	ggggtgtgactggtggtaaa
β-actin	gcctgacggacaggtcat	accgcaagattccataccc

2.5.Luciferase Constructs, Expression Constructs and Transient Transfections

D-box15x-Luc contains fifteen multimerized copies of the cry1a D-box 59-aagttatacaacagc-39 cloned into pGL3Basic (Promega) vector. ARE-Luc contains three multimerized copies of the antioxidant responsive element (ARE) from the yeast GST (fw: tcgagcttggaaatgacattgctaatggtgacaaagcaactttc; rev: tcgagaaagttgctttgtcacc) cloned into pGL3Basic (Promega) vector. Sod-Luc (– 953 Sod1-Luc) contains 1.2 Kb in total, 885bp upstream from the transcription start site and 182bp after the end of first exon (141bp). The fragment containing 1,208bp of the 5′ flanking region of sod1 was subcloned into pGL3basic vector (Promega) upstream to the luciferase gene. For that, a primer with Kpn1 recognition site (fw: ccaccggtacctaacattcttcgaag) were design and used in combination with other primer with a Xho1 recognition site (rev: cgcacagcaaattctcgagcaattg). The resulting product was subcloned into the pGL3-easy vector (Promega) and sequenced. Sod genes were amplified by PCR using Pfu polymerase. LRRcry1a-Luc and LRRper2-Luc was previously described elsewhere (Vatine et al., 2009; Mracek et al., 2010)

All PAR bZip transcription factors expression constructs (Vatine et al., 2009; Ben-Moshe et al., 2010), TEF-1, TEF-2, HLF-1, HLF-2, DBP-1, DBP-2, were based on the CMV promoter driven expression vector pCS2-MTK. Transfections were performed using the FuGene HD (Promega) reagents according to the manufacturer's protocols.

2.6.Real-Time Bioluminescence Assay and Data Analysis

All real-time bioluminescence assays were performed and analyzed as described previously (Vatine et al., 2009, Vallone et al., 2004) using an TopCount NXT (Packard) under various lighting conditions. Luciferase activity was monitored and compared with that of vehicle-treated control cells.

2.7.In vitro Bioluminescence Assay and Data Analysis

PAC-2 cells were plated at a density of 1.25×10^5 cells per well in a 24-well plate (CELLSTAR, Greiner Bio-One). 24 hours later, cells were cotransfected with 250 ng of sod reporters together with 50 ng of β-galactosidase expression vector (to normalize transfection efficiency) and 5 ng of TEF-1, TEF-2, HLF-1, HLF-2, DBP-1 or DBP-2

transcription factors. Luciferase activity was measured using the Luciferase Assay System kit (Promega) and firefly luciferase as a cosubstrate, transfection efficiency was calculated with β-galactosidase activity assay (Sambrook et al., 1989).

2.8.Western Blotting

Cells were plated at a $6x10^5$ density in a 6-well plate. Protein extracts were prepared by sampling cells in 1x Laemmli buffer. The samples were electrophoresed on a SDS polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Binding of the antibodies was visualized using the Pierce-ECL detection system (Thermo Scientific). The primary antibodies pJNK, Pp38 and ERK were purchased from Cell Signaling and vinculin antibody from Sigma Aldrich. Vinculin was used as a loading control for normalization of the samples. Autoradiographic images were quantified with Image Lab™ Software (Bio-Rad).

2.9. DCF-DA assay (ROS levels)

Cells were plated at $3x10^4$ density in a 96-well plate, left two days in DD for desynchronization of the clock and then treated with 250 μM of Cu for 0, 5, 10, 15, 30 minutes and 1 hour, 2, 4, 6, 8 hours. After exposure, cells were incubated with 10 μM of DCF-DA (2',7' – dichlorofluorescin diacetate) in fresh culture medium, (45 min, 26°C, protected from light), washed with PBS one time and suspended in 250 μL of PBS. Fluorescence was measured at 488/530 nm.

2.10. Statistical analyses

For all the assays three independent experiments were performed. The results were expressed as means ± standard error of the mean. Data distribution was tested and parametric (one-way ANOVA) tests were performed, followed by Dunnett's Multiple Comparison post-test. Effects of Cu exposure were verified by the comparison of the control non-treated group. P-values lower than 0.05 were considered statistically significant.

3. Results

3.1.Selection of the copper concentration, induction of cell defense against oxidative stress and sod endogenous gene induction

Based on previous researches with other fish cell lines (Manzl et al., 2004; Rau et al., 2004; Tan et al., 2008; Sandrini et al., 2009; Concu et al., 2017), PAC-2 toxicity-screening

tests were performed with different $CuSO_4$ concentrations ranging from 60 μM to 350 μM in two periods of exposure, 24 and 48 hours (Fig 1a and 1b), cell viability and proliferation were assessed with the MTT assay. The working concentration should not induce pronounced toxicity or increase cell metabolism, therefore, based on these results, the 250 μM concentration of $CuSO_4$ was selected as the LOAEL (lowest observed adverse effect level) concentration.

Next, to access if the 250 μM copper sulfate treatment activated the cellular mechanism against oxidative stress and the duration of this activation, real time bioluminescence assays of transfected PAC-2 cells with the Luc-ARE reporter were performed. The assay showed that immediately after $CuSO_4$ exposure ARE-Luc induction started. But it's only in twenty-four hours after the exposure that a strong activation of ARE-Luc can be detected. Finally, 7 days after the exposure the detected levels of bioluminescence of the copper sulfate treated cells returned to the same levels of bioluminescence of the control cells (Fig 1c).

To further confirm that SOD1 was also induced by copper sulfate and what was the earliest time when it occurred, PAC-2 cells were maintained for 2 days in DD prior to the exposure to 250 μM of $CuSO_4$ and cells were sampled for qRT-PCR in six different time-points: 0 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 9 hours after Cu exposure. The results showed up-regulation of sod1 expression began nine hours after the Cu exposure (Fig 1d). Lastly, as several studies point to the existence of SOD circadian oscillation (Wilking et al., 2013), to investigated if $CuSO_4$ exposure could disturb patterns of sod1 expression in 12:12 light/dark conditions, cells were plated and left 2 days in DD. $CuSO_4$ were then added to the medium in a final concentration of 250 μM in DD. After 24 hours of $CuSO_4$ exposure in DD the cells were entrained for 2 days in LD conditions and then sampled on the third day for qRT-PCR at: 3 hours, 9 hours, 15 hours and 21 hours after the lights went on. The results exhibited that PAC-2 cells had higher sod1 expression between ZT9 and ZT15, that corresponds to the end of the light period and beginning of the dark. Further, $CuSO_4$ exposure interfered with sod1 rhythms by changing amplitude and the time of sod1 peak expression (Fig. 1e)

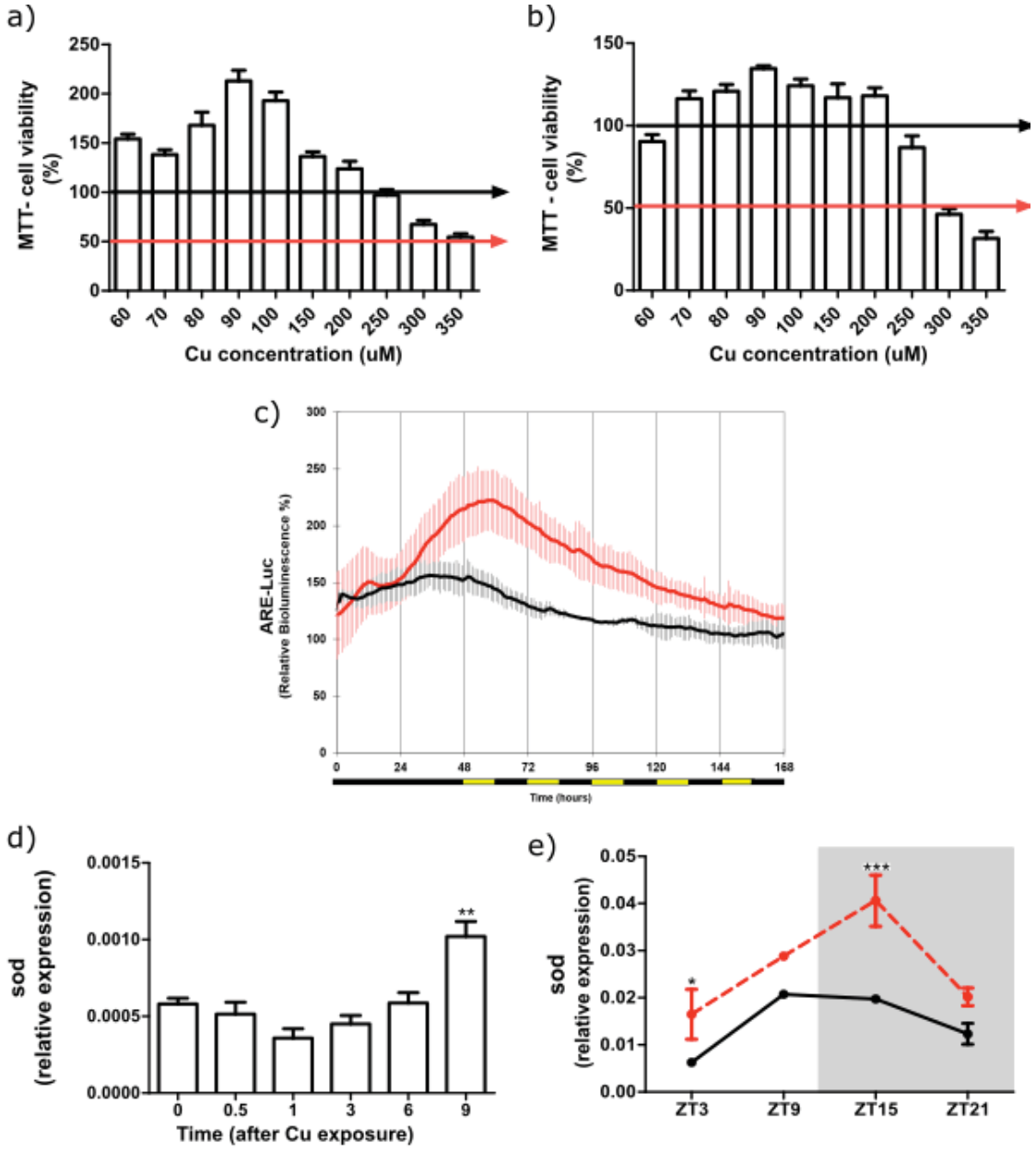


FIGURE 1: MTT, induction of antioxidant response and endogenous expression of sod after treatment with $CuSO_4$. a) and b) MTT assay in PAC-2 cells to assess cell viability and metabolism after a) 24 hours and b) 48 hours of Cu exposure. Relative cell viability (%) are plotted on the y-axis and the $CuSO_4$ concentrations on the x-axes. The results are plotted as the means of three independent experiments performed in triplicate, +/− SD. c) Real time bioluminescence assays of PAC-2 cells transfected with ARE-Luc and treated with 250 µM $CuSO_4$ (red line) or a negative control treated with vehicle (black line). Bioluminescence is plotted on the y-axis and time (hours) on the x-axis. Each time-point represents the mean of at least three independent samples. Black (dark) and yellow (light) bars below the panels represent the different lighting conditions. d) qRT-PCR analysis of the expression of the sod1 gene in PAC-2 under DD conditions treated with 250 µM Cu. Relative mRNA levels are plotted on the y-axis and time in hours after the $CuSO_4$ exposure on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Asterisks indicate effects in comparison to control "0" (**$p<0.01$). e) qRT-PCR analysis of the expression of the sod1 gene in PAC-2 entrained in LD conditions treated with 250 µM Cu (red line) or negative control treated with vehicle (black). Relative mRNA levels are plotted on the y-axis and ZT times on the x-axes. The results are plotted as the means of three independent experiments +/− SD.

3.2.Sod1 Promoter cloning and analysis

For better understanding of the sod1 regulation Sod-Luc (– 953sod1-Luc) was analyzed using the MatInspector (Cartharius et al., 2005) for the presence of enhancer elements responsible from clock-controlled transcription factors and ARE of XRE elements, at matrix similarity of ≥ 0.85. The analysis showed one potential ARE and one E-box region, one HLF and DBP and five TEF/VBP and E4BP4 binding sites (Fig. 2a).

Since, oxidative stress, light and clock elements appeared to be involved on regulation of sod1 in zebrafish, real time bioluminescence assays were performed. PAC-2 cells transfected with the sod-Luc reporter were treated with 250 μM $CuSO_4$ and 300 μM of H_2O_2 and kept under 2 days under DD conditions before monitoring under LD conditions. The result showed robust rhythms of sod-Luc under LD conditions (Fig. 2b). Moreover, copper treatment attenuated the rhythm for several days, while exposure to H_2O_2 only showed an effect on the first twenty-four hours by delaying the peak compared to the control. After, a closer investigation on how light and copper together might have an additive or synergetic effect on the sod-Luc reporter, an in vitro luciferase assay, that allows to quantify the luciferase reporter gene activity by the transfection efficiency using a β-galactosidase assay, was performed. For that, PAC-2 cells transfected with the sod Luc reporter were left in DD conditions for 2 days before exposure to 8 hours of constant light and/or 32 hours of 250 μM of $CuSO_4$. Light and Cu exposure alone were able to equally induce the sod-Luc reporter, but no additive or synergetic effect of combined $CuSO_4$ exposure and light was detected (Fig, 2c).

Finally, to further investigate if SOD1 regulation occurred via clock-controlled transcriptional factors another in vitro luciferase assay was performed. PAC-2 cells maintained in DD conditions were co-transfected with expression constructs encoding the six PAR/bZIP factors and the sod-Luc reporter and left in DD for two more days before the sampling. TEF and HLF transcription factors showed significant induction of the sod Luc reporter, while DBP had no effect (Fig. 2d).

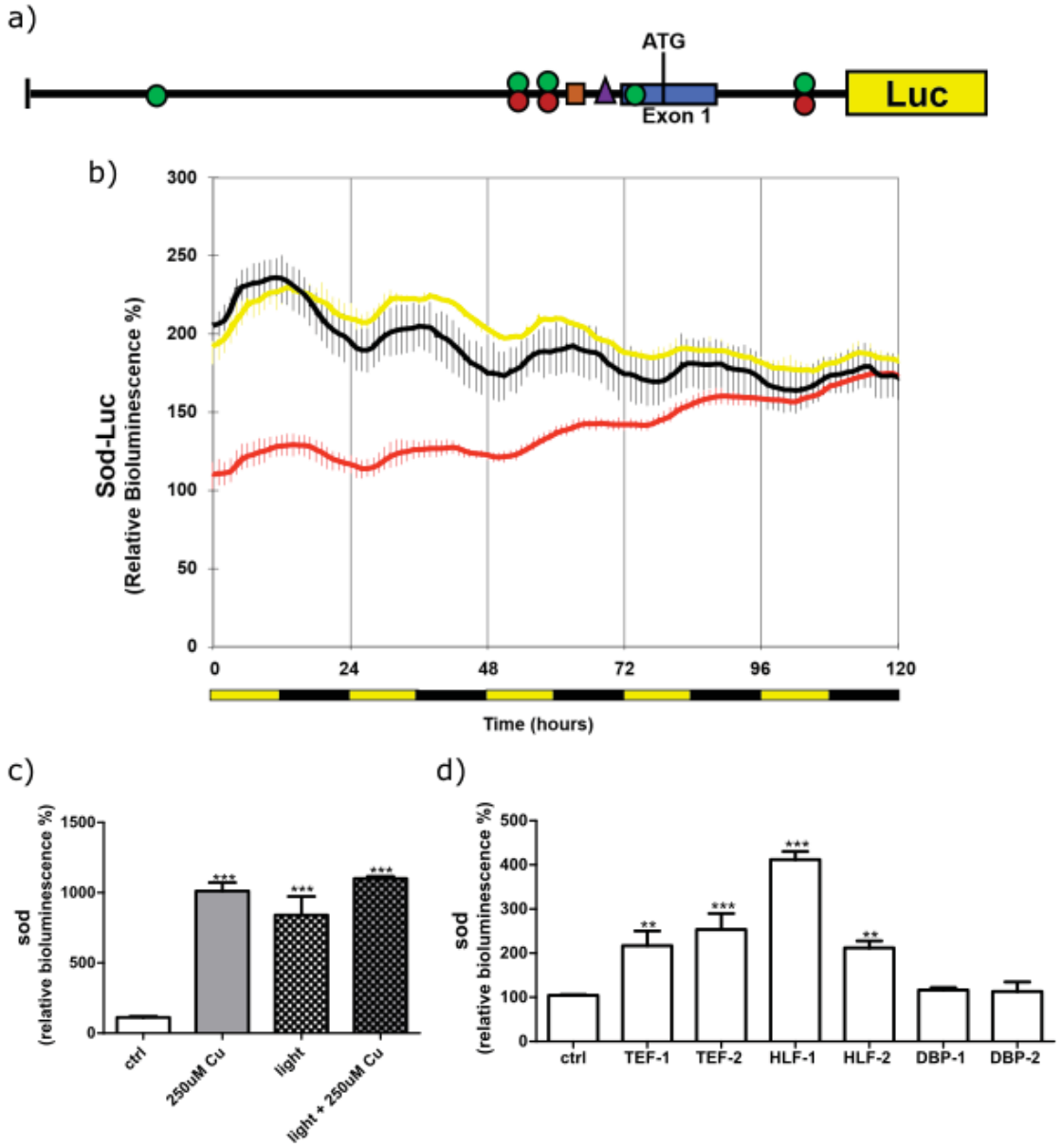


FIGURE 2: Analysis of – 953sod1-Luc promoter. a) Representative figure of the cloned sod1 promoter and the enhancer elements: TEF, VBP, HLF (green circle), E4BP4 (red circle), ARE (brown square) and E-box (purple triangle). b) Real time bioluminescence assays of PAC-2 cells transfected with sod-Luc treated with 250 µM $CuSO_4$ (red line), 300 µM of H_2O_2 (yellow line) or a negative control treated with vehicle (black line). The start of treatments happened shortly before time zero. Bioluminescence is plotted on the y-axis and time (hours) on the x-axis. Each time-point represents the mean of at least three independent samples. Black and yellow bars below the panels represent the different lighting conditions. c) In vitro luciferase assay of PAC-2 cells transfected with the sod-Luc reporter. Cells were left in DD conditions for 2 days before exposure to 8 hours of constant light and/or 32 hours of 250 µM of $CuSO_4$; each exposure regime is indicated below its respective bars. Relative bioluminescence levels (%) are plotted on the y-axis where the highest value measured during the experiment is set arbitrarily as 100%. The results are plotted as the means of three independent experiments performed in triplicate, +/− SD. Each independent experiment was standardized for transfection efficiency using a β-galactosidase assay. Asterisks indicate effects in comparison to control (***$p<0.001$). d) In vitro luciferase assay of PAC-2 cells co-transfected with expression constructs encoding the six PAR/bZip factors and the sod-Luc reporter. Cells were left in DD conditions for 2 days after the co-transfection and then sampled. Each expression construct is indicated below its respective bars. Relative bioluminescence levels (%) are plotted on the y-axis where the highest value measured during the experiment is set arbitrarily as 100%. The results are plotted as the means of three independent experiments performed in triplicate, +/− SD. Each independent experiment was

standardized for transfection efficiency using a β-galactosidase assay. Asterisks indicate effects in comparison to control (**p<0.01, ***p<0.001).

3.3. $CuSO_4$ activation of MAPK signaling pathway and its implications

To evaluate whether the oxidative stress caused by $CuSO_4$ exposure induced cell signaling thought the MAPK pathway in PAC-2, western blotting analysis of phospho-ERK (pERK), phospho-p38 MAPK (pp38), phospho-SAPK/JNK (pJNK) were performed. PAC-2 cells under DD conditions were treated with 250 μM $CuSO_4$ and sampled for 12 hours. To detect the earliest time when the induction occurred and if it persisted for several hours sampling occurred at 0, 5, 10, 15 and 30 minutes and 1, 2, 4, 6, 8 and 12 hours after the $CuSO_4$ treatment. Additional, to connect time activation of pERK, pp38 and pJNK with ROS production DCDF-DA assay were performed to quantify intracellular ROS. The same experimental design used for western blotting was applied for DCDF-DA assay and the measurement occurred after 0, 5, 10, 15 and 30 minutes and 1, 2, 4, 6 and 9 hours after the $CuSO_4$ treatment. Result showed two peaks of greater phosphorylation of pERK, pp38 and pJNK, one between 5 and 30 minutes and another after 4 to 6 after Cu exposure (Fig 3b, 3c and 3d). Likewise, ROS levels displayed the same time trend and were higher at 5 minutes and 30 minutes to 4 hours after $CuSO_4$ exposure (Fig. 3e).

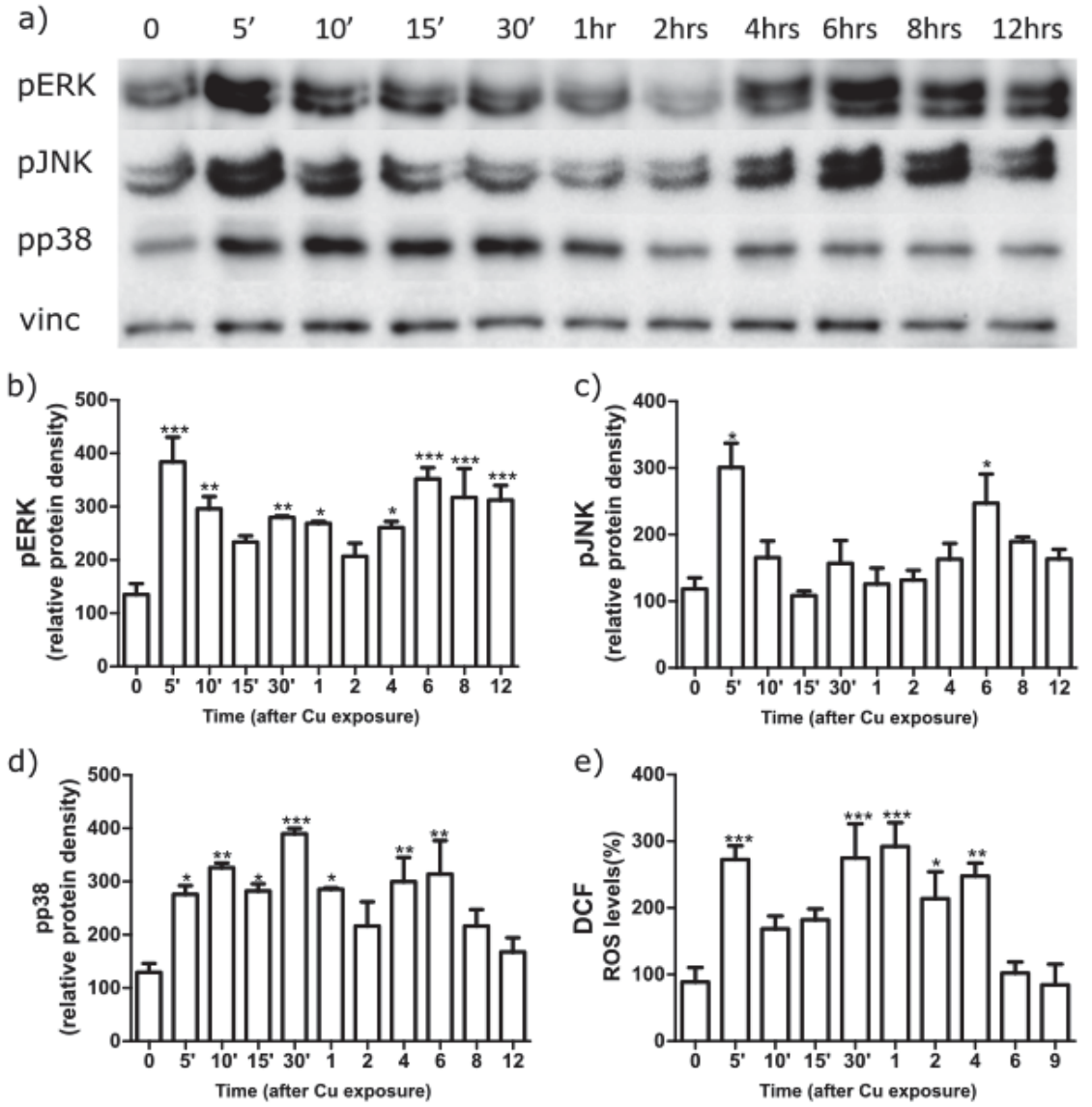


FIGURE 3: Activation of the MAPK via ROS production after $CuSO_4$ exposure. Western blot analysis of phospho-ERK, phospho-p38 MAPK and phospho-SAPK/JNK under DD conditions after 250 µM $CuSO_4$ exposure. a) representative western blots of phospho-ERK (pERK), phospho-p38 MAPK (Pp38), phospho-SAPK/JNK (pJNK) and vinculin in PAC-2 cells during 12 hours of 250uM Cu exposure under DD conditions. In panel a) the duration of the $CuSO_4$ exposure is indicated in minutes (‘) or hours (hrs). These blots are representative of three independent experiments and the final quantification is presented graphically in b) to d). Relative protein density levels (%) are plotted on the y-axis and time in hours after $CuSO_4$ exposure on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Levels of vinculin were used as loading controls for each western blot. e) ROS production in PAC-2 cells after 250 µM Cu exposure. ROS levels (%) are plotted on the y-axis and time in hours after Cu exposure on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Asterisks indicate effects in comparison to control (*p<0.05, **p<0.01, ***p<0.001).

3.4.Effect of $CuSO_4$ exposure on the circadian clock

qRT- PCR was performed to evaluate if exposure to $CuSO_4$ could induce the expression of the per2 and cry1a, known to be light regulated genes. PAC-2 cells were maintained for 2 days in DD prior to the exposure to 250 µM of copper sulfate and cells were sampled

for qRT-PCR in six different time-points: 0 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 9 hours after the copper exposure. The results showed that $CuSO_4$ could induce per2 and cry1a three hours after $CuSO_4$ exposure (Fig 4a and 4b). Also, per2 induction was sustained until the end of the sampling, while cry1a showed a transient induction.

After the confirmation that CuSO4 could induce gene expression of both clock and antioxidant genes, qRT-PCR and real-time bioluminescence assay were used to investigate if $CuSO_4$ exposure could interfere with the clock rhythmicity of both clock, per1, and light-regulated, per2 and cry1a, genes. For both assays with per2 and cry1a cells maintained in DD were treated with 250 μM $CuSO_4$ and, after 24 hours, cells were entrained in LD conditions and sampled at ZT3, ZT9, ZT15 and ZT21 for qRT-PCR or had their bioluminescence monitored for 48 hours. Additionally, to prove that $CuSO_4$ induction observed previously in per2 and cry1a was due to the D-box enhancers, real-time bioluminescence of a reporter containing fifteen multimerized copies of the D-box sequence was used. For that, cells in DD were treated with CuSO4 and then had their bioluminescence monitored during 12 hours of darkness before going through four LD cycles. qRT-PCR (Fig 5c and 5d) and the DAP49 cell line was used to monitor per1 rhythms during LD after exposure to two $CuSO_4$ concentrations (Fig 5e) conditions and if different exposure times in DD conditions, after entrainment in LD, affected per1 rhythmicity in free running conditions (Fig 5f).

It was demonstrated that, both in the endogenous expression and during the real-time assay, while $CuSO_4$ exposure enhanced the rhythms and shifted the peak expression of per2 and cry1a (Fig 4c and 5a; 4d and 5b), it also dampened per1 rhythms (Fig 4e, 5e and 5f). On that sense, for the clock gene per1 the time of the $CuSO_4$ exposure had the same dampening effect at ZT3, ZT6 and ZT9 (Fig 5f) and it was shown to be concentration dependent (Fig 5e). Opposingly, the 15xD-box-Luc showed pronounced rhythm enhancement and intense peak shifting during LD cycles in the presence of $CuSO_4$. Besides, $CuSO_4$ induction in DD was later and smaller than H_2O_2 induction (Fig 5c and 5d).

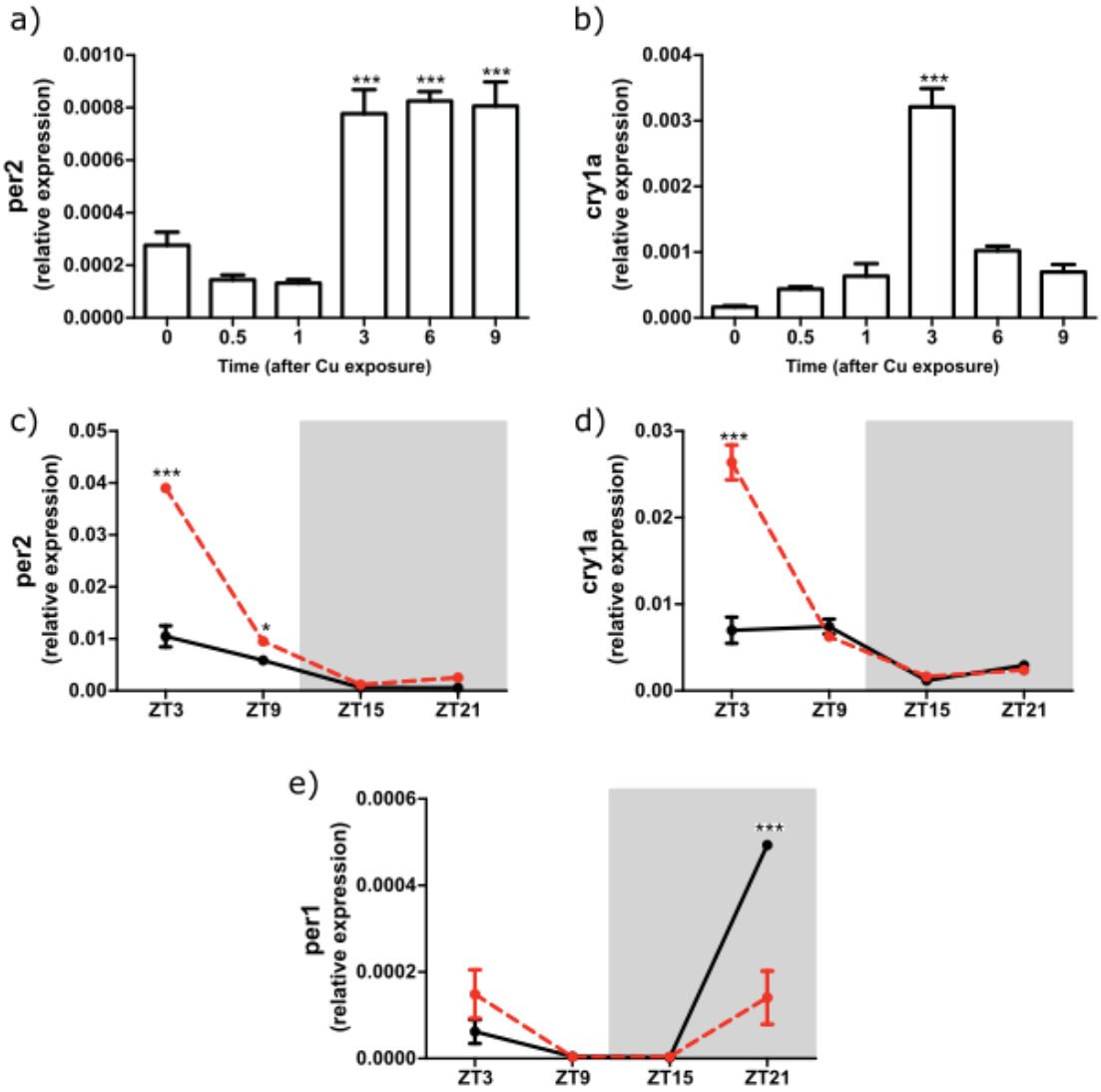


FIGURE 4: Endogenous expression of clock and light regulated genes after $CuSO_4$ exposure. a) and b) qRT-PCR analysis of the expression of the light regulated genes a) per2 and b) cry1a in PAC-2 under DD conditions treated with 250 µM $CuSO_4$. Relative mRNA levels are plotted on the y-axis and time in hours after the $CuSO_4$ exposure on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Asterisks indicate effects in comparison to control "0" (***$p<0.001$). c) to e) qRT-PCR analysis of the expression of the clock regulated gene c) per1 and the light regulated genes d) per2 and e) cry1a in PAC-2 entrained in LD conditions treated with 250 µM $CuSO_4$ (red line) or negative control treated with vehicle (black). Relative mRNA levels are plotted on the y-axis and ZT times on the x-axes. The results are plotted as the means of three independent experiments +/− SD.

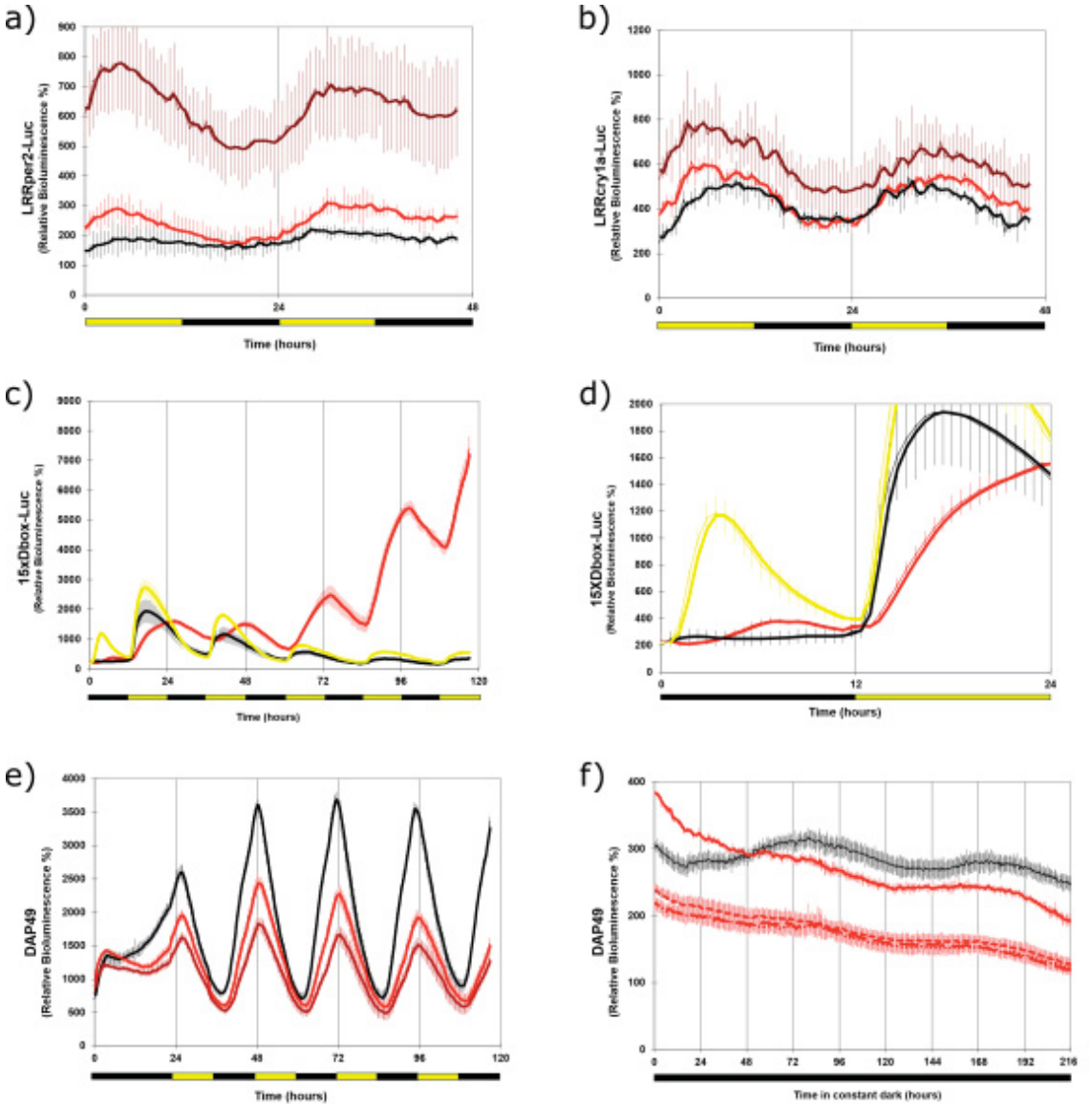


FIGURE 5: Rhythms of clock and light-regulated genes after $CuSO_4$ exposure. Real time bioluminescence assays of PAC-2 cells transfected with a) LRRper2-Luc o b) LRRcry1a-Luc and treated with 300 µM $CuSO_4$ (dark red line) 250 µM $CuSO_4$ (red line) or a negative control treated with vehicle (black line). c) Real time bioluminescence assays of PAC-2 cells transfected with 15xD-box-Luc and treated with 250uM $CuSO_4$ (red line), 300 µM of H_2O_2 (yellow line) or a negative control (black line); d) zoom during 12 hours of DD conditions for comparison between $CuSO_4$ and H_2O_2 induction in DD; e) Real time bioluminescence assays of DAP49 cells treated with 300 µM $CuSO_4$ (dark red line) 250 µM $CuSO_4$ (red line) or a negative control treated with vehicle (black line). f) Real time bioluminescence assays of DAP49 cells treated with 250 µM $CuSO_4$ in different times of the day: ZT0 (continuous red line), ZT3, ZT6 and ZT9 (stippled red line) or a negative control treated with vehicle (black line). Bioluminescence is plotted on the y-axis and time (hours) on the x-axis. Each time-point represents the mean of at least three independent samples. Black (dark) and yellow (light) bars below the panels represent the different lighting conditions.

4. Discussion

4.1.Selection of the copper concentration, induction of cell defense against oxidative stress and sod endogenous gene induction

Copper sulfate is largely used as feed addictive and when added at high dietary levels, between 100 to 250 µg/mL, can increase the growth performance of pigs, chicken and fish (Jacela et al., 2010; EFSA 2012). Also, as Cu ions has an essential role in the growth and survival of cells in culture, medium supplementation with copper sulfate between 50 µM and 100 µM significantly increased the cell survival on mammalian cultures (Qian et al., 2001). That way, it was not a surprise that between 60 µM to 100 µM (Fig 1a and 1b) $CuSO_4$ had and positive effect on PAC-2 cells growth and stimulated higher metabolic rates. For that reason, we chose the 250 µM concentration of copper that hadn't neither positive effect on cells growth, cell viability above 100%, or negative effects, cell death higher than 50%.

This relatively high $CuSO_4$ work concentration may be explained by the embryonic origin of the PAC-2 cells. During the embryonic development, ROS is continuously detected and tend to increase gradually in fish embryo. This occurs as an important immune defense reaction against bacterial infection (Kadomura et al., 2006; Lin et al., 2009). Therefore, it is possible that cell cultures derived from fish embryos are more adapted to a pro-oxidant environment and have a well-developed antioxidant system that allows a rapid clearance of excessive ROS (Lin et al., 2009).

The ARE is a cis-enhancer sequence that mediates the transcriptional activation of several antioxidant enzymes such as SOD, catalase, glutathione peroxidase and glutathione S-transferase (Park and Rho, 2002; Jiang et al., 2015; Boettler et al., 2011). Usually, binding of the transcription factor NF-E2-related factor 2 (Nrf2) to the ARE sequences mediates the induction of the cited enzymes and helps to protect the cell from oxidative damage (Nguyen et al., 2003). It is postulated that metals, including Cu, can activate the Nrf2/ARE pathway (Mattie and Freedman, 2004; Kobayashi et al., 2009), but the correlation between $CuSO_4$ exposure in zebrafish and ARE activation was never successfully demonstrated (Jiang et al., 2015). In this respect, the real-time assay results could, for the first time, show $CuSO_4$ exposure can, not only strongly activate the ARE, but also that this activation can last up to seven days after the acute $CuSO_4$ exposure. Therefore, it is possible to conclude that a one-time copper exposure has long-term effects on the overall antioxidant defense of PAC-2 cells. This corroborates that up-regulation of expression and activity of antioxidant enzymes in aquatic organisms can last up to eleven days after exposure to sub-lethal concentrations of toxic metals (Jo et al., 2008; Yin et al., 2018).

Despite induction of SOD1 and other antioxidant enzymes in fish and mammals by exposure to copper is extensively documented on the literature (Chen et al., 2011; Mela et at., 2013; Jiang et al., 2014; Wang et at., 2015; Simonato et al., 2016), the earliest time after the exposure when this induction occurred is unclear. On this matter, our results showed early up-regulation of sod1 expression less than twelve hours after the $CuSO_4$ exposure. This rapid induction can be extrapolated based on the evidence that mammalian SOD1 can be up-regulated by ARE and xenobiotic responsive element (XRE) enhancers either in combination with or independently when exposed to compounds that generate ROS (Park and Rho, 2002). Although knowledge of XREs or AREs in fish SOD1 is lacking, ROS levels and SOD activity tend to increase gradually over time (Van Tiem and Di Giulio, 2011; Yin et al., 2018).

Several evidences in mammals (Tomás-Zapico et al., 2003; Goncharova et al., 2006; Hardeland et al., 2003) point to the existence of circadian rhythms for SOD1 expression, but detailed studies on fish are still lacking. However, the results for PAC-2 endogenous expression sod1 are consistent with Vera and Migaud (2017) that have found that sod2 expression in atlantic salmon starts to increase during the photic phase and has higher levels at the beginning of the dark period. Besides, as a trend of altered rhythmicity in oxidative stress components have implications in several pathologies (Wilking et al., 2013) and $CuSO_4$ exposure in PAC-2 interfered drastically in sod1 expression levels, it is possible that circadian imbalance caused by exposure to $CuSO_4$ contributes to some diseases. Ultimately, together with the involvement of catalase on the control of light regulated genes (Hirayama et al., 2007), that result raises the question whether sod1 gene is light-regulated and is also connected with the circadian clock machinery.

4.2.Sod1 Promoter cloning and analysis

As previously discussed, ARE regions are responsible for up-regulation of antioxidant defenses when oxidative stress occurs (Kobayashi et al., 2006) and are found on mammalian sod1 promoter (Wang et al., 2007). Here we confirm that the promoter region of sod1 in zebrafish can also recruit Nrf2, who is responsible for binding to ARE, and be up-regulated by oxidative stress (Shi and Zhou, 2010). On the other hand, several binding sites for members of the PAR/bZIP (proline and acidic amino acid-rich basic leucine zipper) transcription factor family were also present on the cloned region of sod1.

Thyrotroph embryonic factor (TEF) or vitellogenin gene–binding protein (VBP), hepatic leukemia factor (HLF) and D-box–binding protein (DBP) accumulate with robust circadian rhythms in mammalian and zebrafish tissues, therefore they are strongly regulated by the core clock (Gachon et al., 2004; Ben-Moshe et al., 2010). Lastly, E4-binding protein 4 (E4BP4) is implicated in complementing the PAR/bZIP factors regulation by competing for the same binding sites. E4BP4 suppress the transcription of target genes, while PAR/bZIP have activation properties (Ben-Moshe et al., 2010).

Binding sites, five in total, for CREB and ATF1 were also found. CREB, and its paralogue ATF1, show circadian variations in mammalian and chicken pineal glands, peaking during the dark period (Ginty et al., 1993; Gau et al.; 2002 Tischkau et al., 2003), but their circadian regulation in zebrafish still remain unconfirmed (Hirayama et al., 2005; Li et al., 2013).

It is important to notice that the Sod-Luc reporter behaved similarly to the sod1 endogenous expression, where during the light phase its activity increased and reached the peak at the beginning of the dark phase. Therefore, along with the *in vitro* bioluminescence assay it was reinforced the hypothesis of sod1 being also light-regulated. In that respect, as TEF/VBP and HLF were shown to be active sites of regulation on the SOD1 promoter, this implies that they can define how light regulates the expression of the gene pointing to SOD1 being tightly coupled with circadian clock function. (Ben-Moshe et al., 2010).

$CuSO_4$ exposure, on the other hand, responsible for Sod-Luc activation and rhythm attenuation, was again confirmed to have the long-term toxic consequences probably because ROS generation continues for several days after the treatment. That behavior is easily understood when comparing to the direct treatment with ROS that had only a transient effect on SOD1 rhythm. At last, $CuSO_4$ exposure showed that ARE is also involved on SOD1 zebrafish regulation and neither ARE or TEF and HLF can surpass the results of each other's regulation.

4.3.$CuSO_4$ activation of MAPK signaling pathway and its implications

It is known that both, light and copper, can induce ROS production, that, by its turn, can activate the MAPK pathway in zebrafish and several mammalian cell cultures (Hirayama et al., 2007; Ravindran et al., 2011; Fanjul-Moles et al., 2013; Chen et al., 2017). The activation of this signaling pathway is trough phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38. Once activated, ERK, JNK, and p38 can phosphorylate many proteins and transcription factors, resulting in the enhancement of the transcriptional activity of numerous genes (Johnson, G. L., & Lapadat, 2002). That way, the activation of MAPK signaling pathway is essential in the light-induced transcriptional activation of clock genes cry1a and per2 (Cermakian et al., 2002; Hirayama et al., 2009; Ramos et al., 2014) and for the activation of antioxidant defenses via ARE/Nrf2 pathway (Shi and Zhou, 2010; Chen et al., 2017; Jeong et al., 2017).

Similarly, ERK induction by $CuSO_4$ exposure on PAC-2 cells also resemble light induction in Z3 cells, that had higher levels documented within 30 min of light exposure (Cermakian et al., 2002; Vatine et al., 2011). Furthermore, previous researches that investigated ROS generation, via metal or xenobiotic exposure, also found activation of p38, JNK and ERK (Samet et al., 1998; Mattie and Freedman, 2004; Shi and Zhou, 2010; Ravindran et al., 2011). Phosphorylation of JNK and p38 normally results in the transcriptional activation of genes involved in stress responses, growth arrest, or apoptosis and, for that reason, oxidative stress tends to activate those proteins in a preferential manner (Mattie et al., 2008). Moreover, there is evidence that ERK, who leads to cell survival and proliferation during oxidant injury, can attenuate both p38 and JNK activity (Marais and Marshall, 1996; Mattie and Freedman, 2004; Shi and Zhou, 2010).

In that sense, since PAC-2 cells show two distinct times of MAPK pathway induction, one right after the copper exposure and another one after 4 hours, the sustained activation of the ERK together with p38 and JNK can lead not only to uncontrolled proliferation and unscheduled cell death (Son et al., 2011) but, this cross-activation suggests that Cu exposure may influence antioxidant response and the circadian regulation in zebrafish.

4.4. Effect of $CuSO_4$ exposure on the circadian clock

It was previously demonstrated that the D-box enhancer mediates light regulated gene expression in fish (Mracek et al., 2012). In addition, exposure to light results in elevated ROS levels that induces D-box mediated transcription (Hirayama et al., 2007). For that reason, ROS production caused by $CuSO_4$ exposure (Manzl et al., 2004; Craig et al., 2007) was responsible for both light-regulated genes induction expression in DD and concentration dependent behavior and peak shifting in LD. Moreover, the real-time assay with the 15xD-box-Luc is a strong evidence that Cu exposure disrupts rhythmicity and expression of per2 and cry1a by inducing D-box enhancer elements.

In addition, although, both cry1a and per2 shared D-box enhancer elements, per2 D-box function is dependent on the presence of a proximal E-box enhancer (Pagano et al., 2017). CRY1A directly interacts with the CLOCK-BMAL complex and assists the establishment and maintenance of a high-amplitude rhythm (Tamai et al., 2007) while PER2 is responsible to maintain the CLOCK:BMAL heterodimer in the cytoplasm resulting in transactivation repression (Hirayama et al., 2003). Because of those differences, per2 sustained activation in DD and higher amplitude when in LD conditions can be partially explained.

Interestingly, $CuSO_4$ induction of sod occurred with a delay in comparison to per2 and cry1a genes, corroborating the existence of the link between the regulation of the redox cellular state and the photic signaling pathways implicated in circadian control previously described by Hirayama et al. (2007).

On the other hand, per1 amplitude dampening and desynchronization of clock transcriptional rhythms was also observed in rats exposed to constant light (Quian et al., 2013). This suggests that acute $CuSO_4$ exposure can have similar effects as extended periods with uninterrupted light exposure.

While ROS plays a key role in light induced gene expression in zebrafish cells and tissues, copper treatment disrupts the redox balance by generating ROS. So, in addition to playing a key role in resetting the clock by light, the redox state of the cell is also a major factor contributing to the maintenance of circadian rhythmicity by the core clock mechanism itself. Our results demonstrated that copper could trigger redox changes and impact on the function of all the evaluated genes, mainly in the core clock.

5. *Conclusion*

The results presented here strongly support a relationship between $CuSO_4$ acute exposure, and consequently increase of the ROS production, activating the MAPK pathway. That leaded to not only activation of antioxidant defense response, but also resulted in impaired cyclical expression, dampened amplitude, and altered phase of genes involved in the circadian clock in PAC-2 cells. Further, it was also demonstrated that zebrafish expression of SOD1 can be regulate either by ARE ligands and PAR/bZIP transcription factors, being SOD1 also sincronized with the circadian clock.

Acknowledgments

This research was supported by DAAD (Research Grants for Doctoral Candidates and Young Academics - scholarship).

References

Ben-Moshe, Z.; Vatine, G.; Alon, S.; Tovin, A.; Mracek, P.; Foulkes, N.S.; Gothilf, Y. Multiple PAR and E4BP4 bZIP transcription factors in zebrafish: diverse spatial and temporal expression patterns. Chronobiology International. 27: 1509–1531. 2010. doi: 10.3109/07420528.2010.510229.

Boettler, U.; Sommerfeld, K.; Volz, N.; Pahlke, G.; Teller, N.; Somoza, V.; Lang, R.; Hofmann, T.; Marko, D. Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression J. Nutr. Biochem., 22: 426-440. 2011. doi: 10.1016/j.jnutbio.2010.03.011.

Cartharius, K.; Frech, K.; Grote, K.; Klocke, B.; Haltmeier, M.; Klingenhoff, A.; Frisch, M.; Bayerlein, M.; Werner, T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics, 21:2933-2942. 2005. doi: 10.1093/bioinformatics/bti473

Cermakian, N.; Pando, M.P.; Thompson, C.L.; Pinchak, A.B.; Selby, C.P.; Gutierrez, L.; Wells, D.A.; Cahill, G.M.; Sancar, A.; Sassone-Corsi, P. Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. Current Biology 12: 844-848. 2002. doi: 10.1016/S0960-9822(02)00835-7.

Chen, D.; Zhang, D.; Jimmy, C.Y.; Chan, K. M. Effects of Cu_2O nanoparticle and $CuCl_2$ on zebrafish larvae and a liver cell-line. Aquatic toxicology, 105: 344-354. 2011. doi: 10.1016/j.aquatox.2011.07.005.

Chen, Z.; Wan, X.; Hou, Q.; Shi, S.; Wang, L.; Chen, P.; Zhu, X.; Zeng, C.; Qin, W.; Zhou, W.; Liu, Z. GADD45B mediates podocyte injury in zebrafish by activating the ROS-GADD45B-p38 pathway. Cell death & disease, 7: e2068. 2017. doi: 10.1038/cddis.2015.300.

Cheng, W.; Tung, Y.H.; Liu, C.H.; Chen, J.C. Molecular cloning and characterisation of cytosolic manganese superoxide dismutase (cytMn-SOD) from the giant freshwater prawn Macrobrachium rosenbergii. Fish & shellfish immunology, 20: 438-449. 2006. doi: 10.1016/j.fsi.2005.05.016.

Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques. 15:532-534, 536-537. 1993.

Concu, R.; Kleandrova, V.V.; Speck-Planche, A.; Cordeiro, M.N.D. Probing the toxicity of nanoparticles: a unified in silico machine learning model based on perturbation theory. Nanotoxicology, 1-16. 2017. doi: 10.1080/17435390.2017.1379567

Craig, P.M.; Wood, C.M.; Mcclelland, G.B. Oxidative stress response and gene expression with acute copper exposure in zebrafish (Danio rerio). American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 293:1882-1892. 2007. doi: 10.1152/ajpregu.00383.2007.

Dröge, W. Free radicals in the physiological control of cell function. Physiological Reviews. 82: 47–95. 2002. doi: 10.1152/physrev.00018.2001.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. EFSA Journal 10:2969, 38 pp. 2012. Available on: www.efsa.europa.eu/efsajournal

Fanjul-Moles, M.L. ROS signaling pathways and biological rhythms: perspectives in crustaceans. Frontiers in bioscience. 18:665-675. 2013. doi: 10.2741/4129.

Gachon, F.; Fonjallaz, P.; Damiola, F.; Gos, P.; Kodama, T.; Zakany, J.; Duboule, D.; Petit, B.; Tafti, M.; Schibler, U. The loss of circadian PAR bZip transcription factors results in epilepsy. Genes & development, 18:1397-1412. 2004. doi: 10.1101/gad.301404

Gau, D.; Lemberger, T.; von Gall, C.; Kretz, O.; Le Minh, N.; Gass, P.; Schmid, W.; Schibler, U.; Korf, H.W.; Schutz, G. Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock. Neuron 34:245–253. 2002. doi: 10.1016/S0896-6273(02)00656-6.

Ginty, D.D.; Kornhauser, J.M.; Thompson, M.A.; Bading, H.; Mayo, K.E.; Takahashi, J.S.; Greenberg, M. E. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science. 260:238-238. 1993. doi: 10.1126/science.8097062.

Goncharova, N.D.; Shmaliy, AV.; Bogatyrenko, T.N.; Koltover, V.K. Correlation between activity of antioxidant enzymes and circadian rhythms of corticosteroids in Macaca mulatta monkeys of different age. Exp. Gerontol. 41:778–783. 2006. doi: 10.1016/j.exger.2006.04.006.

Harris, Z. L.; Gitlin, J. D. Genetic and molecular basis for copper toxicity. The American journal of clinical nutrition. 63:836S-841S. 1996.

Hardeland, R.; Coto-Montes, A.; Poeggeler, B. Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. Chronobiology International. 20:921–962. 2003. doi: 10.1081/CBI-120025245.

Helsel, M.E.; Franz, K.J. Pharmacological activity of metal binding agents that alter copper bioavailability. Dalton Transactions. 44:8760-8770. 2015. doi: 10.1039/c5dt00634a.

Hirayama, J.; Cardone, L.; Doi, M.; Sassone-Corsi, P. Common pathways in circadian and cell cycle clocks: light-dependent activation of Fos/AP-1 in zebrafish controls CRY-1a and WEE-1. Proceedings of the National Academy of Sciences of the United States of America, 102:10194-10199. 2005. doi: 10.1073/pnas.0502610102

Hirayama, J.; Cho, S.; Sassoni-Corsi, P. Circadian control by the reduction/oxidation pathway: Catalase represses light-dependent clock gene expression in the zebrafish. Proceedings of the National Academy of Sciences of the United States of America, 104:15747–15752. 2007. doi: 10.1073/pnas.0705614104.

Hirayama, J.; Fukuda, I.; Ishikawa, T.; Kobayashi, Y.; Todo, T. New role of zCRY and zPER2 as regulators of sub-cellular distributions of zCLOCK and zBMAL proteins. Nucleic Acids Res 31:935–943. 2003. doi: 10.1093/nar/gkg174

Hirayama, J.; Miyamura, N.; Uchida, Y.; Asaoka, Y.; Honda, R.; Sawanobori, K.; Todo, T.; Yamamoto, T.; Sassone-Corsi, P.; Nishina, H. Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish. Cell Cycle. 8:2794-2801. 2009. doi: 10.4161/cc.8.17.9447.

Jacela, J.Y.; DeRouchey, J.M.; Tokach, M.D.; Goodband, R.D.; Nelssen, J.L.; Renter, D.G.; Dritz, S.S. Feed additives for swine: fact sheets–high dietary levels of copper and zinc for young pigs, and phytase. Journal of Swine Health and Production, 18: 87-91. 2010. doi: 10.4148/2378-5977.7068

Jeong, C.B.; Kang, H.M.; Lee, M.C.; Kim, D.H.; Han, J.; Hwang, D.S.; Souissi, S.; Lee, S.; Shin, K.; Park,H.G.; Lee, J.S. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. Scientific reports. 7:41323. 2017. doi: 10.1038/srep41323.

Jiang, W.D.; Liu, Y.; Hu, K.; Jiang, J.; Li, S.H.; Feng, L.; Zhou, X.Q. Copper exposure induces oxidative injury, disturbs the antioxidant system and changes the Nrf2/ARE (CuZnSOD) signaling in the fish brain: protective effects of myo-inositol. Aquatic toxicology, 155:301-313. 2014. doi: 10.1016/j.aquatox.2014.07.003.

Jiang, W.D.; Liu, Y.; Jiang, J.; Wu, P.; Feng, L.; Zhou, X.Q. Copper exposure induces toxicity to the antioxidant system via the destruction of Nrf2/ARE signaling and caspase-3-regulated DNA damage in fish muscle: amelioration by myo-inositol. Aquatic Toxicology. 159:245-255. 2015. doi: 10.1016/j.aquatox.2014.12.020.

Jiménez-Ortega, V.; Cano-Barquilla, P.; Scacchi, P.; Cardinali, D.P.; Esquifino, A.I. Cadmium-Induced Disruption in 24-h Expression of Clock and Redox Enzyme Genes in Rat Medial Basal Hypothalamus: Prevention by Melatonin. Frontiers in Neurology. 2:13. 2011. doi:10.3389/fneur.2011.00013

Jo, P.G.; Choi, Y.K.; Choi, C.Y. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, Crassostrea gigas in response to cadmium exposure. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 147:460-469. 2008. doi: 10.1016/j.cbpc.2008.02.001.

Johnson, G. L.; Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science, 298:1911-1912. 2002. doi: 10.1126/science.1072682.

Kadomura, K.; Nakashima, T.; Kurachi, M.; Yamaguchi, K.; Oda, T. Production of reactive oxygen species (ROS) by devil stinger (Inimicus japonicus) during embryogenesis. Fish & shellfish immunology, 21: 209-214. 2006. doi: 10.1016/j.fsi.2005.11.006

Kobayashi, A.; Kang, M.I.; Watai, Y.; Tong, K.I.; Shibata, T.; Uchida, K.; Yamamoto, M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol. Cell. Biol. 261:221-229. 2006. doi: 10.1128/MCB.26.1.221-229.2006.

Kobayashi, M.; Li, L.; Iwamoto, N.; Nakajima-Takagi, Y.; Kaneko, H.; Nakayama, Y.; Eguchi, M.; Wada, Y.; Kumagai, Y.; Yamamoto, M. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. Molecular and cellular biology, 29:493-502.2009. doi: 10.1128/MCB.01080-08

Krishnan, N.; Davis, A.J.; Giebultowicz, J.M. Circadian regulation of response to oxidative stress in Drosophila melanogaster. Biochemical and Biophysical Research Communications. 374:299–303. 2008. doi: 10.1016/j.bbrc.2008.07.011.

Li, D.; Ueta, E.; Kimura, T.; Yamamoto, T.; Osaki, T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. Cancer science. 95:644-650. 2004. doi: 10.1111/j.1349-7006.2004.tb03323.x

Li, Y.; Li, G.; Wang, H.; Du, J.; Yan, J. Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. PLoS computational biology. 9: e1002940. 2013. doi: 10.1371/journal.pcbi.1002940.

Lin, C.T.; Tseng, W.C.; Hsiao, N.W.; Chang, H.H.; Ken, C.F. Characterization, molecular modelling and developmental expression of zebrafish manganese superoxide dismutase. Fish & shellfish immunology, 27:318-324. 2009. doi:10.1016/j.fsi.2009.05.015

Lin, S.; Gaiano, N.; Culp, P.; Burns, J.C.; Friedmann, T.; Yee, J.K.; Hopkins, N. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science 265: 666–669. 1994. doi: 10.1126/science.8036514.

Livingstone, D.R. Contaminant-stimulates reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin.42:656-666. 2001. doi: 10.1016/S0025-326X(01)00060-1.

Manzl, C.; Enrich, J.; Ebner, H.; Dallinger, R.; Krumschnabel, G. Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. Toxicology, 196: 57-64. 2004. doi: 10.1016/j.tox.2003.11.001

Marais, R.; Marshall, C.J. Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv. 27:101–125. 1996.

Mattie, M.D.; Freedman, J.H. Copper-inducible transcription: regulation by metal-and oxidative stress responsive pathways. American Journal of Physiology-Cell Physiology, 286:C293-C301. 2004. doi: 10.1152/ajpcell.00293.2003

Mattie, M.D.; McElwee, M.K.; Freedman, J.H. Mechanism of copper-activated transcription: activation of AP-1, and the JNK/SAPK and p38 signal transduction pathways. Journal of Molecular Biology. 383:1008-1018. 2008. doi: 10.1016/j.jmb.2008.08.080.

McElwee, M.K.; Song, M.O.; Freedman, J.H. Copper activation of NF-κB signaling in HepG2 cells. Journal of Molecular Biology. 393:1013-1021. 2009. doi: 10.1016/j.jmb.2009.08.077.

Mela, M.; Guiloski, I.C.; Doria, H.B.; Rabitto, I.S.; da Silva, C.; Maraschi, A.C.; Prodócimo, V.; Freire, C.A.; Randi, M.A.F.; de Assis, H.C.S. Risks of waterborne copper exposure to a cultivated freshwater Neotropical catfish (Rhamdia quelen). Ecotoxicology and Environmental Safety, 88:108–16. 2013. doi:10.1016/j.ecoenv.2012.11.002

Milani, P.; Gagliardi, S.; Cova, E.; Cereda, C. SOD1 transcriptional and posttranscriptional regulation and its potential implications in ALS. Neurology research international, 2011:9 pages. doi: 10.1155/2011/458427

Mracek, P.; Santoriello, C.; Idda, M.L.; Pagano, C.; Ben-Moshe, Z.; Gothilf, Y.; Vallone, D.; Foulkes, N. S. (2012). Regulation of per and cry Genes Reveals a Central Role for the D-Box Enhancer in Light-Dependent Gene Expression. PLoS ONE. 7(12). 2012. doi: 10.1371/journal.pone.0051278.

Nguyen, T.; Sherratt, P.J.; Pickett, C.B. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annual review of pharmacology and toxicology, 43:233-260. 2003. doi: 10.1146/annurev.pharmtox.43.100901.140229.

Nordberg, J.; Arnér, E.S.J. Reactive oxygen species antioxidants and the mammalian thioredoxin system. Free Radical Biology and Medicine. 31:1287-1311. 2001. doi: 10.1016/S0891-5849(01)00724-9.

O'Neill, J.S.; Feeney, K.A. Circadian Redox and Metabolic Oscillations in Mammalian Systems. Antioxidants & Redox Signaling, 20:2966–2981. 2014. doi: 10.1089/ars.2013.5582.

Ostrakhovitch, E.A.; Lordnejad, M.R.; Schliess, F.; Sies, H.; Klotz, L.O. Copper ions strongly activate the phosphoinositide-3-kinase/Akt pathway independent of the

generation of reactive oxygen species. Archives of biochemistry and biophysics, 397:232-239. 2002. doi: 10.1006/abbi.2001.2559.

Pagano, C.; Ceinos, R.M.; Vallone, D.; Foulkes, N.S. The Fish Circadian Timing System: The Illuminating Case of Light-Responsive Peripheral Clocks. In: Biological Timekeeping: Clocks, Rhythms and Behaviour (pp. 177-192). Springer, New Delhi. 2017.

Park, E.Y.; Rho, H.M. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. Molecular and cellular biochemistry, 240:47-55. 2002.

Patel, S.; Velingkaar, N.S.; Kondratov, R.V. Transcriptional control of antioxidant defense by the circadian clock. Antioxidants & Redox Signaling. 20:2997–3006. 2014. doi: 10.1089/ars.2013.5671.

Pham, A.N.; Xing, G.; Miller, C.J.; Waite, T.D. Fenton-like copper redox chemistry revisited: Hydrogen peroxide and superoxide mediation of copper-catalyzed oxidant production. Journal of catalysis. 301:54-64. 2013. doi: 10.1016/j.jcat.2013.01.025.

Qian, Y.; Khattak, S.F.; Xing, Z.; He, A.; Kayne, P.S.; Qian, N.X.; Pan, S.; Li, Z. J. Cell culture and gene transcription effects of copper sulfate on Chinese hamster ovary cells. Biotechnology progress. 27:1190-1194. 2001. doi: 10.1002/btpr.630.

Qian, J.; Block, G.D.; Colwell, C.S.; Matveyenko, A.V. Consequences of exposure to light at night on the pancreatic islet circadian clock and function in rats. Diabetes, 62:3469-3478. 2013. doi: 10.2337/db12-1543

Ramos, B.C.; Moraes, M.N.C.; Poletini, M.O.; Lima, L.H.; Castrucci, A.M.L. From blue light to clock genes in zebrafish ZEM-2S cells. PLoS One. 9:e106252. 2014. doi: 10.1371/journal.pone.0106252.

Rau, M.A.; Whitaker, J.; Freedman, J.H.; Di Giulio, R. T. Differential susceptibility of fish and rat liver cells to oxidative stress and cytotoxicity upon exposure to prooxidants. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 137:335-342. 2004. doi: 10.1016/j.cca.2004.03.001.

Ravindran, J.; Gupta, N.; Agrawal, M.; Bhaskar, A.B.; Rao, P.L. Modulation of ROS/MAPK signaling pathways by okadaic acid leads to cell death via, mitochondrial mediated caspase-dependent mechanism. Apoptosis. 16:145-161. 2011. doi: 10.1007/s10495-010-0554-0.

Rutter, J.; Reick, M.; McKnight, S.L. Metabolism and the control of circadian rhythms. Annual Review of Biochemistry, 71:307–331. 2002. doi: 10.1146/annurev.biochem.71.090501.142857.

Sambrook, J.; Fritsch, E.F.; Maniatis, T. Molecular cloning: a laboratory manual (Ed. 2). Cold spring harbor laboratory press. 1989

Samet, J.M.; Graves, L.M.; Quay, J.; Dailey, L.A.; Devlin, R.B.; Ghio, A.J.; Wu, W.; Bromberg, P.A.; Reed, W. Activation of MAPKs in human bronchial epithelial cells

exposed to metals. Am J Physiol Lung Cell Mol Physiol 275: L551–L558, 1998. doi: 10.1152/ajplung.1998.275.3.L551.

Sandrini, J.Z.; Bianchini, A.; Trindade, G.S.; Nery, L.E.M.; Marins, L.F.F. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish (Danio rerio) ZFL cells. Aquatic toxicology, 95: 285-291. 2009. doi:10.1016/j.aquatox.2009.02.016

Santos, E.M.; Ball, J.S.; Williams, T.D.; Wu, H.; Ortega, F.; Van Aerle, R.; Katsiadaki, I.; Falciani, F.; Viant, M.R.; Chipman, J.K.; Tyler, C.R. Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. Environmental science & technology, 44:820-826. 2009. doi: 10.1021/es902558k.

Shi, X.; Zhou, B. The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. Toxicological Sciences, 115:391-400. 2010. doi: 10.1093/toxsci/kfq066.

Simonato, J. D.; Mela, M.; Doria, H.B.; Guiloski, I.C.; Randi, M.A.F.; Carvalho, P.S.; Meletti, P.C.; Silva de Assis, H.C.; Bianchini, A.; Martinez, C.B.R. Biomarkers of waterborne copper exposure in the Neotropical fish Prochilodus lineatus. Aquatic Toxicology. 170:31-41. 2015. doi: 10.1016/j.aquatox.2015.11.012.

Son, Y.; Cheong, Y.K.; Kim, N.H.; Chung, H.T.; Kang, D.G.; Pae, H.O. Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? Journal of signal transduction. 2011: 792639. 2011. doi: 10.1155/2011/792639

Song, M.O.; Li, J.; Freedman, J.H. Physiological and toxicological transcriptome changes in HepG2 cells exposed to copper. Physiological Genomics. 38:386-401. 2009. doi: 10.1152/physiolgenomics.00083.2009.

Storz, P. Forkhead homeobox type O transcription factors in the responses to oxidative stress. Antioxidants & redox signaling. 14:593-605. 2011. doi: 10.1089/ars.2010.3405.

Tamai, T.K.; Young, L.C.; Whitmore, D. Light signaling to the zebrafish circadian clock by Cryptochrome 1a. Proceedings of the National Academy of Sciences of the United States of America. 104:14712–14717. 2007. doi: 10.1073/pnas.0704588104.

Tan, F.; Wang, M.; Wang, W.; Lu, Y. Comparative evaluation of the cytotoxicity sensitivity of six fish cell lines to four heavy metals in vitro. Toxicology in vitro, 22:164-170. 2008. doi: 10.1016/j.tiv.2007.08.020

Tischkau, S.A.; Mitchell, J.W.; Tyan, S.H.; Buchanan, G.F.; Gillette, M.U. Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. Journal of Biological Chemistry. 278:718-723. 2003. doi: 10.1074/jbc.M209241200

Tomás-Zapico, C.; Coto-Montes, A.; Martínez-Fraga, J.; Rodríguez-Colunga, M.J.; Tolivia, D. Effects of continuous light exposure on antioxidant enzymes, porphyric enzymes and cellular damage in the Harderian gland of the Syrian hamster. J Pineal Res. 34:60-68. 2003. doi: 10.1034/j.1600-079X.2003.02951.x.

Tseng, H.L.; Li, C.J.; Huang, L.H.; Chen, C.Y.; Tsai, C.H.; Lin, C.N.; Hsu, H.Y. Quercetin 3-O-methyl ether protects FL83B cells from copper induced oxidative stress through the PI3K/Akt and MAPK/Erk pathway. Toxicology and Applied Pharmacology. 264:104-113. 2012. doi: 10.1016/j.taap.2012.07.022.

Turski, M.L.; Brady, D.C.; Kim, H.J.; Kim, B.E.; Nose, Y.; Counter, C.M.; Thiele, D.J. A novel role for copper in Ras/mitogen-activated protein kinase signaling. Molecular and Cellular Biology, 32:1284-1295. 2012. doi: 10.1128/MCB.05722-11.

Valko, M.; Morris, H.; Cronin, M.T.D. Metals, Toxicity and oxidative stress. Current Medicinal Chemistry. 12:1161-1208. 2005. doi: 10.2174/0929867053764635.

Vallone, D.; Gondi, S.B.; Whitmore, D.; Foulkes, N.S. E-box function in a period gene repressed by light. Proceedings of the National Academy of Sciences of the United States of America. 101: 4106–4111. 2004. doi: 10.1073/pnas.0305436101.

Vallone, D.; Santoriello, C.; Gondi, S.B.; Foulkes, N.S. Basic protocols for zebrafish cell lines: maintenance and transfection. Circadian Rhythms: Methods and Protocols, 429-441. 2007. doi: 10.1007/978-1-59745-257-1_35.

Van Tiem, L.A.; Di Giulio, R. T. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE-and ARE-associated gene induction in zebrafish (Danio rerio). Toxicology and applied pharmacology. 254:280-287. 2011. doi: 10.1016/j.taap.2011.05.002.

Vatine, G.; Vallone, D.; Appelbaum, L.; Mracek, P.; Ben-Moshe, Z.; Lahiri, K.; Gothilf, Y.; Foulkes, N.S. Light directs zebrafish period2 expression via conserved D and E boxes. PLoS Biol 7: e1000223. 2009. doi: 10.1371/journal.pbio.1000223.

Vatine, G.; Vallone, D.; Gothilf, Y.; Foulkes, N.S. It's time to swim! Zebrafish and the circadian clock. Febs Letters. 585:1485-1494. 2011. doi: 10.1016/j.febslet.2011.04.007.

Vera, L.M.; Migaud, H. Hydrogen peroxide treatment in Atlantic salmon induces stress and detoxification response in a daily manner. Chronobiology international. 33:530-542. 2016. doi: 10.3109/07420528.2015.1131164.

Volpato, G. L.; Trajano, E. Biological rhythms. In: Fish Physiology: The Physiology of Tropical Fishes: The Physiology of Tropical Fishes. p.21-101. 2005.

Wang, B.; Feng, L.; Jiang, W.D.; Wu, P.; Kuang, S.Y.; Jiang, J.; Tang, L.; Tang, W.N.; Zhang, Y.A.; Liu, Y.; Zhou, X.Q. Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: preventive role of arginine. Aquatic toxicology, 158:125-137. 2015. doi: 10.1016/j.aquatox.2014.10.025.

Wang, X.; Tomso, D.J.; Chorley, B.N.; Cho, H.Y.; Cheung, V.G.; Kleeberger, S.R.; Bell, D.A. Identification of polymorphic antioxidant response elements in the human genome. Human molecular genetics. 16:1188-1200. 2007. doi: 10.1093/hmg/ddm066.

Wang, X.J.; Hayes, J.D.; Higgins, L.G.; Wolf, C.R.; Dinkova-Kostova, A.T. Activation of the NRF2 signaling pathway by copper-mediated redox cycling of para-and ortho-

hydroquinones. Chemistry & Biology. 17:75-85. 2010. doi: 10.1016/j.chembiol.2009.12.013.

Wendelaar Bonga, S.E. The Stress Response in Fish. Physiological Reviews. 77: 591-620. 1997.

Wilking, M.; Ndiaye, M.; Mukhtar, H.; Ahmad, N. Circadian rhythm connections to oxidative stress: implications for human health. Antioxidants & redox signaling, 19:192-208. 013. doi: 10.1089/ars.2012.4889.

Wu, L.; Reddy, A.B. Rethinking the clockwork: redox cycles and non-transcriptional control of circadian rhythms. Biochemical Society Transactions, 42:1–10. 2014. doi:10.1042/BST20130169.

Zavitsanos, K.; Nunes, A.M.; Malandrinos, G.; Hadjiliadis, N. Copper effective binding with 32–62 and 94–125 peptide fragments of histone H2B. Journal of inorganic biochemistry. 105:102-110. 2011. doi: 10.1016/j.jinorgbio.2010.09.002.

Zelinski, E. L.; Deibel, S. H.; Mcdonald, R.J. The trouble with circadian clock dysfunction: multiple deleterious effects on the brain and body. Neuroscience and Behavioral Reviews, 40:80–101. 2014. doi: 10.1016/j.neubiorev.2014.01.007.

Zelko, I.N.; Mariani, T.J.; Folz, R.J. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology and Medicine, 33:337-349. 2002. doi: 10.1016/S0891-5849(02)00905-X.

Yin, J.; Wang, A.P.; Li, W.F.; Shi, R.; Jin, H.T.; Wei, J.F. Time-response characteristic and potential biomarker identification of heavy metal induced toxicity in zebrafish. Fish & shellfish immunology. 72:309-317. 2018. doi: 10.1016/j.fsi.2017.10.047.

7. DISCUSSÃO GERAL E CONSIDERAÇÕES FINAIS

Os resultados apresentados nesta tese fornecem as primeiras informações a respeito dos mecanismos biológicos através do qual a exposição ao sulfato de cobre induz a defesa antioxidante celular e desregula a expressão de genes ligados ao controle da ritmicidade circadiana em peixes *Danio rerio* e células PAC-2.

No primeiro capítulo, observou-se que, de modo geral, as oscilações diárias tanto das enzimas antioxidantes SOD e CAT, quanto das proteínas chave responsáveis pela regulação do relógio circadiano, per1, per2 e cry1a, são perturbadas pela exposição a níveis naturalmente encontrados de sulfato de cobre.

O resultado vem de encontro a pesquisas que propõe que baixíssimas concentrações de íons de cobre em água doce, já causam respostas biológicas e efeitos

adversos aos organismos expostos (GRNC, 2002; CRAIG et al., 2007; MELA et al., 2013; SIMONATO et al., 2016; SILVA ACOSTA et al., 2016). Ademais, estudos comportamentais em zebrafish e peixes teleósteos expostos a concentrações realísticas de Cu mostram que concentrações tão baixas quanto 7 μg/L até 40 μg/L podem diminuir a aptidão do animal no meio ambiente (SLOMAN et al., 2003; VIEIRA et al 2009; DA SILVA ACOSTA et al., 2016). Porém, é a primeira vez que resultados apontam que a sincronização do organismo com o ambiente pode estar prejudicada frente a exposição a um composto gerador de ROS. Isso é reforçado uma vez que todos os genes relativos ao controle da ritmicidade circadiana, endógeno (per1) e regulados pela luz (per2 e cry1a), apresentam padrões de expressão distintos do descrito para situação ideal e do encontrado na literatura para o *D. rerio* (VELARDEA et al., 2010; MRACEK et al., 2012; AMARAL & JOHNSTON, 2012; PEYRIC et al., 2013).

No presente estudo pôde-se ainda observar que os efeitos do Cu variaram significativamente entre os dois órgãos analisados, estando de acordo com a literatura que descreve que parâmetros oxidativos dependem do tecido (MACHADO et al., 2013; QU et al., 2014; DA SILVA ACOSTA et al., 2016). Isso se deve provavelmente à distribuição dos íons de cobre aos diferentes tecidos, quando este é absorvido pelo organismo.

A coordenação entre as enzimas SOD e CAT é perdida nos dois tecidos analisados quando da exposição às concentrações de sulfato de cobre acima do permitido pelas leis nacionais e internacionais. Mais especificamente, sabe-se que SOD é a primeira linha de defesa das células ao radical superóxido (VAN DER OOST et al., 2003; HALLIWELL & GUTTERIDGE, 2007) e variações rítmicas de atividade dessa enzima já foram reportadas em diversos organismos (ALBARRÁN et al., 2001; HARDELAND et al., 2003; MARTIN et al., 2003; GONCHAROVA et al., 2006; SUBRAMANIAN et al., 2008) porém nunca em um peixe teleósteo. Dessa forma, para a SOD foi possível correlacionar a maior atividade da enzima com maiores níveis de ATP intracelular. Isso acontece, pois, alimentação, locomoção, atividade cerebral e o ciclo sono/vigília, modulam a intensidade dos processos metabólicos durante o dia (HARDELAND et al., 2003; PATEL et al., 2014). Ainda, evidência de ritmo ultradiano de sua atividade foi detectada.

Para CAT foi possível correlacionar os resultados com a ritmicidade circadiana de per2 e cry1a, genes que dependem da ativação da luz, nos dois tecidos analisados. Esse

resultado fornece dados para a consolidação da hipótese da produção de peróxido de hidrogênio por uma oxidase fototransdutora contendo flavina “in vivo” (HIRAYAMA et al., 2007).

Em última análise, as correlações das enzimas antioxidantes com elementos do relógio circadiano permitiram a continuação do estudo dos mecanismos de controle da transcrição da SOD e da CAT, apresentados no capítulo 2.

De forma geral, confrontando as três concentrações de sulfato de cobre entre si é possível estabelecer que as perturbações nos padrões de 24 horas são concentração dependentes, pois com o aumento da concentração de íons de Cu na água mais parâmetros avaliados, assim como mais perturbações nos padrões desses parâmetros foram identificados. Entretanto, o aumento das concentrações não revela o princípio concentração-resposta quando não se leva em conta o horário das coletas e a avaliação se dá de forma geral. Também, não apenas foi possível concluir que a coleta de espécimes em diferentes horários do dia pode afetar a mensuração de parâmetros bioquímicos relacionados a defesas antioxidantes, mas também a criteriosa observação do horário de coleta e o conhecimento dos padrões oscilatórios dos parâmetros que se deseja avaliar deverão ser sempre observados a fim de produzir melhores trabalhos de pesquisa na área da toxicologia.

Assim, embora os metais tenham múltiplos efeitos conhecidos nos sistemas biológicos, e dentre eles a capacidade de interferir com a defesa antioxidante (VALKO et al., 2005; JAISHANKAR et al., 2014), um efeito pouco estudado é o seu papel na programação da expressão gênica. Ainda que o cádmio já tenha sido descrito como um agente perturbador da ritmicidade de per1, per2 e cry1a e de alguns parâmetros da defesa antioxidante em ratos (JIMENÉZ-ORTEGA et al., 2011) e que a exposição de ratos à fumaça do cigarro altera a expressão de genes envolvidos no controle da ritmicidade circadiana (GEBEL et al., 2006), nenhum dos estudos anteriores caracterizou qual o mecanismo subjacente entre os xenobióticos pró-oxidantes, o sistema circadiano e o antioxidante.

Sendo assim, o segundo capítulo apresentado focou no entendimento das bases moleculares subjacentes à interferência do sulfato de cobre no controle da transcrição tanto das enzimas SOD e CAT quanto dos genes envolvidos no controle da ritmicidade circadiana.

Os resultados apresentados no segundo capítulo mostram uma relação entre a exposição aguda de Cu e, consequentemente, o aumento da produção de ROS, que ativou a via da MAPK. Tanto a luz quanto o Cu são capazes de aumentar a produção de ROS e ativar a via da MAPK (OSTRAKHOVITCH et al., 2002; HIRAYAMA et al., 2007; MATTIE et al., 2008; SONG & FREEDMAN, 2009; MCELWEE et al., 2009; WANG et al., 2010; TSENG et al., 2012; TURSKI et al., 2012) foi evidente que esse evento levou não apenas a ativação da resposta à defesa antioxidante, mas também resultou em distúrbios da expressão cíclica, amplitude amortecida e fase alterada dos genes envolvidos no relógio circadiano em células PAC-2.

A intensificação da reposta da SOD1 se deu tanto através da região ARE presente no promotor da enzima, que é responsável pela elevação da regulação das defesas antioxidantes quando o estresse oxidativo ocorre (KOBAYASHI et al., 2006), quanto pela presença de vários locais de ligação para fatores de transcrição fortemente regulados pelo relógio circadiano e pela luz (GACHON et al., 2004; BEN-MOSHE et al., 2010). Entre esses, o fator de leucemia hepática (HLF) e o fator embrionário de tirotropo (TEF), também conhecido como proteína de ligação ao gene da vitelogenina (VBP) são sítios ativos de regulação no promotor da SOD1; isso implica que a luz também é um fator que regula a expressão do gene (BEN-MOSHE et al., 2010). Sendo ele sincronizado à função de relógio.

Ainda, as repostas biológicas obtidas em fígado, um importante órgão de biotransformação e alvo de diversos xenobióticos, no primeiro capítulo e os resultados obtidos com a linhagem PAC-2 mostram significa similaridade. Sendo que frente à exposição ao $CuSO_4$, enquanto per1 exibe diminuição da amplitude, per2 e cry1a apresentam a situação contrária. Em relação a SOD o padrão normal de ritmicidade na expressão gênica tanto em fígado quanto em PAC-2 são os mesmos. Isso sinaliza que a cultura celular PAC-2 pode ser empregada como um modelo alternativo à testes em animais para o estudo de efeitos tóxicos de contaminantes.

Concluindo, pela promoção da geração de estresse oxidativo, e, como consequência, por intermédio da ativação da via da MAPK, a expressão de genes responsáveis pela marcação de fase, cry1a, per2 e per1, e genes sincronizados à função do relógio, como o sod1, tem sua ritmicidade normal comprometida. Assim, o presente estudo é o primeiro a mostrar que a exposição a concentrações de $CuSO_4$ comumente encontradas em

ambientes com atividade antrópica podem prejudicar a sincronização do organismo com o ambiente.

8. BIBLIOGRAFIA GERAL

AEBI, H. Catalase in vitro. **Methods in Enzymology**, v. 105, p. 121–126, 1984.

ALBARRÁN, M.T.; LÓPEZ-BURILLO, S.; PABLOS, M.I.; REITER, R.J.; AGAPITO, M.T. Endogenous rhythms of melatonin, total antioxidant status and superoxide dismutase activity in several tissues of chick and their inhibition by light. **Journal of Pineal Research**, v. 30, p. 227–233, 2001.

ALBRECHT, U. The mammalian circadian clock: A network of gene expression. **Frontiers in Bioscience**, v. 9, p. 48–55, 2004.

AMARAL, I.P.G. & JOHNSTON, I.A. Circadian expression of clock and putative clock-controlled genes in skeletal muscle of the zebrafish. **American Journal of Physiology. Regulatory, Integrative and Comparative Physiology**, v. 302, p. R193–206, 2012

BALSALOBRE, A.; DAMIOLA, F.; SCHIBLER, U. A serum shock induces circadian gene expression in mammalian tissue culture cells. **Cell**, v. 93, p. 929–937, 1998.

BEN-MOSHE, Z.; VATINE, G.; ALON, S.; TOVIN, A.; MRACEK, P.; FOULKES, N.S.; GOTHILF, Y. Multiple PAR and E4BP4 bZIP transcription factors in zebrafish: diverse spatial and temporal expression patterns. **Chronobiology International**, v. 27, p. 1509–1531. 2010.

BOETTLER, U.; SOMMERFELD, K.; VOLZ, N.; PAHLKE, G.; TELLER, N.; SOMOZA, V.; LANG, R.; HOFMANN, T.; MARKO, D. Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. **Journal of Nutritional Biochemistry**, v. 22, p. 426-440, 2011.

BRADFORD, M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BRIGGS, J. P. The zebrafish: a new model organism for integrative physiology. **American Journal of Physiology: Regulatory Integrative and Comparative Physiology,** v. 282, p. R3–R9, 2002.

CAHILL, G. M. Clock mechanisms in zebrafish. **Cell and Tissue Research**, v. 309, p. 27–34, 2002.

CARR, A. J.; WHITMORE, D. Imaging of single light- responsive clock cells reveal fluctuating free-running periods. **Nature Cell Biology,** v. 7, p. 319–321, 2005.

CARTHARIUS, K.; FRECH, K.; GROTE, K.; KLOCKE, B.; HALTMEIER, M.; KLINGENHOFF, A.; FRISCH, M.; BAYERLEIN, M.; WERNER, T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. **Bioinformatics**, v. 21, p. 2933-2942, 2005.

CAVALLARI, N.; FRIGATO, E.; VALLONE, D.; FROHLICH, N.; LÓPEZ- OLMEDA, J. F.; FOA, A.; BERTI, R.; SÁNCEZ-VÁZQUEZ, F. J.; BERTOLUCCI, C.; FOULKES, N. S. A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. **PLoS Biology**, v. 9, p. e1001142, 2011.

CECON, E.; FLORES, D. E. F. L. Regulação da expressão gênica nas engrenagens do relógio circadiano me mamíferos. **Revista da Biologia – USP**, v. 4, p. 28–33, 2010.

CERMAKIAN, N.; PANDO, M.P.; THOMPSON, C.L.; PINCHAK, A.B.; SELBY, C.P.; GUTIERREZ, L.; WELLS, D.A.; CAHILL, G.M.; SANCAR, A.; SASSONI-CORSI, P. Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. **Current Biology,** v. 12, p. 844-848, 2002.

CHEN, D.; ZHANG, D.; JIMMY, C.Y.; CHAN, K. M. Effects of Cu_2O nanoparticle and $CuCl_2$ on zebrafish larvae and a liver cell-line. **Aquatic Toxicology**, v. 105, p. 344-354, 2011

CHEN, Z.; WAN, X.; HOU, Q.; SHI, S.; WANG, L.; CHEN, P.; ZHU, X.; ZENG, C.; QIN, W.; ZHOU, W.; LIU, Z. GADD45B mediates podocyte injury in zebrafish by activating the ROS-GADD45B-p38 pathway. **Cell Death & Disease**, v. 7, p. e2068, 2017.

CHENG, W.; TUNG, Y.H.; LIU, C.H.; CHEN, J.C. Molecular cloning and characterisation of cytosolic manganese superoxide dismutase (cytMn-SOD) from the

giant freshwater prawn Macrobrachium rosenbergii. **Fish & Shellfish Immunology**, v. 20, p. 438-449, 2006.

CHO, H.; ZHAO, X.; HATORI, M.; YU, R.T.; BARISH, G.D.; LAM, M.T.; CHONG, L.W.; Di TACCIO, L.; ATKINS, A.R.; GLASS, C.K.; LIDDLE, C.; AUWERX, J.; DOWNES, M.; PANDA, S.; EVANS, R.M. Regulation of Circadian Behavior and Metabolism by Rev-erbα and Rev-erbβ. **Nature**, v. 485, p. 123–127, 2012.

CHOMCZYNSKI, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. **Biotechniques**, v. 15, p. 532-534, 536-537, 1993.

CONAMA – Conselho Nacional do Meio Ambiente, 2005. Resolução no. 357, de 17 de março de 2005. Ministério do Meio Ambiente, 23p. 2005.

CONCU, R.; KLEANDROVA, V.V.; SPECK-PLANCHE, A.; CORDEIRO, M.N.D. Probing the toxicity of nanoparticles: a unified in silico machine learning model based on perturbation theory. **Nanotoxicology**, p. 1-16, 2017.

CRAIG, P.M.; WOOD, C.M.; MCCLELLAND G.B. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). **American Journal of Physiology. Regulatory, Integrative and Comparative Physiology**, v. 293, p. 1882-1892, 2007.

D'CRUZ, S.C.; JUBENDRADASS, R.; MATHUR, P.P. Bisphenol A induces oxidative stress and decreases levels of insulin receptor substrate 2 and glucose transporter 8 in rat testis. **Reproductive Sciences**, v. 19, p. 163-172, 2012.

DRÖGE, W. Free radicals in the physiological control of cell function. **Physiological Reviews,** v. 82, p. 47–95, 2002.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. **EFSA Journal**, v. 10, 38 pp, 2012. Available on: www.efsa.europa.eu/efsajournal

EMERY, P.; REPPERT, S. M. A rhythmic Ror. **Neuron**, v. 43, p. 443–446, 2004.

FANJUL-MOLES, M.L. ROS signaling pathways and biological rhythms: perspectives in crustaceans. **Frontiers in Bioscience**, v. 18, p. 665-675, 2013.

GACHON, F.; FONJALLAZ, P.; DAMIOLA, F.; GOS, P.; KODAMA, T.; ZAKANY, J.; DUBOULE, D.; PETIT, B.; TAFTI, M.; SCHIBLER, U. The loss of circadian PAR bZip transcription factors results in epilepsy. **Genes & development**, v. 18, p. 1397-1412. 2004.

GAU, D.; LEMBERGER, T.; VON GALL, C.; KRETZ, O.; LE MINH, N.; GASS, P.; SCHMID, W.; SCHIBLER, U.; KORF, H.W.; SCHUTZ, G. Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock. **Neuron**, v. 34, p. 245–253, 2002.

GEBEL, S.; GERSTMAYER, B.; KUHL, P.; BORLAK, J.; MEURRENS, K.; MULLER, T. The kinetics of transcriptomic changes induced by cigarette smoke in rat lungs reveals a specific program of defense, inflammation, and circadian clock gene expression. **Toxicological Sciences,** v. 93, p. 422–431, 2006.

GINTY, D.D.; KORNHAUSER, J.M.; THOMPSON, M.A.; BADING, H.; MAYO, K.E.; TAKAHASHI, J.S.; GREENBERG, M. E. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. **Science**, v. 260, p. 238-238, 1993.

GONCHAROVA, N.D.; SHMALIY, A.V.; BOGATYRENKO, T.N.; KOLTOVER, V.K. Correlation between activity of antioxidant enzymes and circadian rhythms of corticosteroids in *Macaca mulatta* monkeys of different age. **Experimental Gerontology**, v. 41, p. 778–783, 2006.

GRNC. **Risque pour l'environnement–Evaluation des risques associes aux rejets chimiques des installations nucleaires du Nord-Cotentin**. Groupe de Radioecologie du Nord Cotentin, pp. 210, 2002.

HALLIWELL, B.; GUTTERIDGE. **Free Radicals in Biology and Medicine**. 4ed. Nova York: Oxford University Press, 2007.

HARDELAND, R.; COTO-MONTES, A.; POEGGELER, B. Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. **Chronobiology International**, v. 20, p. 921–62, 2003.

HARRIS, Z. L.; GITLIN, J. D. Genetic and molecular basis for copper toxicity. **The American Journal of Clinical Nutrition**, v. 63, p. 836S-841S, 1996.

HASTINGS, M.; O'NEILL, J. S.; MAYWOOD, E.S. Circadian clocks: regulators of endocrine and metabolic rhythms. **Journal of Endocrinology**, v. 195, p. 187-198, 2007.

HELSEL, M.E.; FRANZ, K.J. Pharmacological activity of metal binding agents that alter copper bioavailability. **Dalton T**, v. 44, p. 8760-8770, 2015.

HEM, J.D. **Study and interpretation of the chemical characteristics of natural water**. v. 2254. Department of the Interior, US Geological Survey. 1985.

HILL, A. J.; TERAOKA, H.; HEIDEMAN, W.; PETERSON, R. E. Zebrafish as a model vertebrate for investigating chemical toxicity. **Toxicological Sciences: An Official Journal of the Society of Toxicology**, v. 86, p. 6–19, 2005.

HIRAYAMA, J.; CARDONE, L.; DOI, M.; SASSONE-CORSI, P. Common pathways in circadian and cell cycle clocks: light-dependent activation of Fos/AP-1 in zebrafish controls CRY-1a and WEE-1. **Proceedings of the National Academy of Sciences of the United States of America**, v. 102, p. 10194-10199, 2005.

HIRAYAMA, J.; CHO, S.; SASSONI-CORSI, P. Circadian control by the reduction/oxidation pathway: Catalase represses light-dependent clock gene expression in the zebrafish. **Proceedings of the National Academy of Sciences of the United States of America**, v. 104, p. 15747–15752, 2007.

HIRAYAMA, J.; FUKUDA, I.; ISHIKAWA, T.; KOBAYASHI, Y.; TODO, T. New role of zCRY and zPER2 as regulators of sub-cellular distributions of zCLOCK and zBMAL proteins. **Nucleic Acids Research**, v. 31, p. 935–943, 2003.

HIRAYAMA, J.; MIYAMURA, N.; UCHIDA, Y.; ASAOKA, Y.; HONDA, R.; SAWANOBORI, K.; TODO, T.; YAMAMOTO, T.; SASSONE-CORSI, P.; NISHINA, H. Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish. **Cell Cycle**, v. 8, p. 2794-2801, 2009.

HOSKINS, L. J.; VOLKOFF, H. Daily patterns of mRNA expression of two core circadian regulatory proteins, Clock2 and Per1, and two appetite-regulating peptides, OX

and NPY, in goldfish (*Carassius auratus*). **Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology**, v. 163, p. 127–136, 2012.

HOOVEN, L. A.; SHERMAN, K. A.; BUTCHER, S.; GIEBULTOWICZ, J. M. Does the clock make the poison? Circadian variation in response to pesticides. **PLoS ONE**, v. 4, p. e6469, 2009.

HULAK, M.; GAZO, I.; SHALIUTINA, A.; LINHARTOVA, P. In vitro effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, v. 158, p. 64-71, 2013.

HUR, S.P.; TAKEUCHI, Y.; ITOH, H.; UCHIMURA, M.; TAKAHASHI, K.; KANG, H. C.; LEE, Y.D.; KIM, S.J.; TAKEMURA, A. Fish sleeping under sandy bottom: Interplay of melatonin and clock genes. **General and Comparative Endocrinology**, v. 177, p. 37–45, 2012.

IDDA, M. L.; BERTOLUCCI, C.; VALLONE, D.; GOTHILF, Y.; SÁNCHEZ-VÁZQUEZ, F. J.; FOULKES, N. S. Circadian clocks: lessons from fish. **Progress in Brain Research**, v. 199, p. 41–57, 2012.

JACELA, J.Y.; DEROUCHEY, J.M.; TOKACH, M.D.; GOODBAND, R.D.; NELSSEN, J.L.; RENTER, D.G.; DRITZ, S.S. Feed additives for swine: fact sheets–high dietary levels of copper and zinc for young pigs, and phytase**. Journal of Swine Health and Production**, v. 18, p. 87-91, 2010.

JAISHANKAR, M.; TSETEN, T.; ANBALAGAN, N.; MATHEW, B. B.; BEEREGOWDA, K.N. Toxicity, mechanism and health effects of some heavy metals. **Interdisciplinary Toxicology**, v. 7, p. 60–72, 2014.

JEONG, C.B.; KANG, H.M.; LEE, M.C.; KIM, D.H.; HAN, J.; HWANG, D.S.; SOUISSI, S.; LEE, S.; SHIN, K.; PARK,H.G.; LEE, J.S. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. **Scientific reports**, v. 7, p. 41323, 2017.

JIANG, W.D.; LIU, Y.; HU, K.; JIANG, J.; LI, S.H.; FENG, L.; ZHOU, X.Q. Copper exposure induces oxidative injury, disturbs the antioxidant system and changes the Nrf2/ARE (CuZnSOD) signaling in the fish brain: protective effects of myo-inositol. **Aquatic toxicology**, v. 155, p. 301-313, 2014.

JIANG, W.D.; LIU, Y.; JIANG, J.; WU, P.; FENG, L.; ZHOU, X.Q. Copper exposure induces toxicity to the antioxidant system via the destruction of Nrf2/ARE signaling and caspase-3-regulated DNA damage in fish muscle: amelioration by myo-inositol. **Aquatic Toxicology**, v. 159, p. 245-255, 2015.

JIMÉNEZ-ORTEGA, V.; CANO-BARQUILLA, P.; SCACCHI, P.; CARDINALI, D.P.; ESQUIFINO, A.I. Cadmium-Induced Disruption in 24-h Expression of Clock and Redox Enzyme Genes in Rat Medial Basal Hypothalamus: Prevention by Melatonin. **Frontiers in Neurology**, v. 2, 2011.

JIN, Y.; ZHANG, X.; SHU, L.; CHEN, L.; SUN, L.; QIAN, H.; LIU, W.; FU, Z. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). **Chemosphere**, v. 78, p. 846–852, 2010.

JO, P.G.; CHOI, Y.K.; CHOI, C.Y. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,** v. 147, p. 460-469, 2008.

JOHNSON, G. L.; LAPADAT, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. **Science**, v. 298, p. 1911-1912, 2002.

KADOMURA, K.; NAKASHIMA, T.; KURACHI, M.; YAMAGUCHI, K.; ODA, T. Production of reactive oxygen species (ROS) by devil stinger (Inimicus japonicus) during embryogenesis. **Fish & Shellfish Immunology**, v. 21, p. 209-214, 2006.

KARAN, V.; VITOROVIC, S.; TUTUNDZIC, V.; POLEKSIC, V. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. **Ecotoxicology and Environmental Safety**, v. 40, p. 49–55, 1998.

KO, C.H.; TAKAHASHI, J.S. Molecular components of the mammalian circadian clock. **Human Molecular Genetics**, v. 15, p. 271–277, 2006.

KOBAYASHI, A.; KANG, M.I.; WATAI, Y.; TONG, K.I.; SHIBATA, T.; UCHIDA, K.; YAMAMOTO, M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. **Molecular and Cellular Biology**, v. 261, p. 221-229, 2006.

KOBAYASHI, M.; LI, L.; IWAMOTO, N.; NAKAJIMA-TAKAGI, Y.; KANEKO, H.; NAKAYAMA, Y.; EGUCHI, M.; WADA, Y.; KUMAGAI, Y.; YAMAMOTO, M. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. **Molecular and Cellular Biology**, v. 29, p. 493-502, 2009.

KOBAYASHI, Y.; ISHIKAWA, T.; HIRAYAMA, J.; DAIYASU, H.; KANAI, S.; TOH, H.; FUKUDA, I.; TSUJIMURA, T.; TERADA, N.; KAMEI, Y.; YUBA, S.; IWAI, S.; TODO, T. Molecular analysis of zebrafish photolyase/cryptochrome family: Two types of cryptochromes present in zebrafish. **Genes to Cells**, v. 5, p. 725–738, 2000.

KONDRATOV, R.V. A role of the circadian system and circadian proteins in aging. **Ageing Research Reviews**, v. 6, p. 12–27, 2007.

KONO, Y. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. **Archives of Biochemistry and Biophysics**, v. 186, p. 189-195, 1978.

KRISHNAN, N.; DAVIS, A.J.; GIEBULTOWICZ, J.M. Circadian regulation of response to oxidative stress in *Drosophila melanogaster*. **Biochemical and Biophysical Research Communications**, v. 374, p. 299–303, 2008.

KULCZYKOWSKA, E.; POPEK, W.; B.G., KAPOOR. **Biological clock in fish**. 1ed. CRC Press, 2010

LEREBOURS, A.; GONZALEZ, P.; ADAM, C.; CAMILLERI, V.; BOURDINEAUD, J.P.; GARNIER-LAPLACE, J. Comparative analysis of gene expression in brain, liver, skeletal muscles, and gills of zebrafish (*Danio rerio*) exposed to environmentally relevant waterborne uranium concentrations. **Environmental Toxicology and Chemistry**, v. 28, p. 1271-1278, 2009.

LI, D.; UETA, E.; KIMURA, T.; YAMAMOTO, T.; OSAKI, T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. **Cancer Science**, v. 95, p. 644-650, 2004.

LI, M.; ZHENG, Y.; LIANG, H.; ZOU, L.; SUN, J.; ZHANG, Y.; QIN F.; LIU, S.; WANG, Z. Molecular cloning and characterization of cat, gpx1 and Cu/Zn-sod genes in pengze crucian carp (*Carassius auratus* var. Pengze) and antioxidant enzyme modulation induced by hexavalent chromium in juveniles. **Comparative Biochemistry and Physiology. Toxicology & Pharmacology**, v. 157, p. 310–321, 2013.

LI, Y.; LI, G.; WANG, H.; DU, J.; YAN, J. Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. **PLoS Computational Biology**, v. 9, p. e1002940, 2013.

LIN, C.T.; TSENG, W.C.; HSIAO, N.W.; CHANG, H.H.; KEN, C.F. Characterization, molecular modelling and developmental expression of zebrafish manganese superoxide dismutase. **Fish & Shellfish Immunology**, v. 27, p. 318-324, 2009.

LIN, S.; GAIANO, N.; CULP, P.; BURNS, J.C.; FRIEDMANN, T.; YEE, J.K.; HOPKINS, N. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. **Science**, v. 265, p. 666–669, 1994.

LIVINGSTONE, D. R. Contaminant-stimulates reactive oxygen species production and oxidative damage in aquatic organisms. **Marine Pollution Bulletin**, v. 42, p. 656-666, 2001.

LÓPEZ-OLMEDA, J.F.; EGEA-ÁLVAREZ, M. SÁNCHEZ-VÁZQUEZ, F. J. Glucose tolerance in fish: Is the daily feeding time important? **Physiology and Behavior**, v. 96, p. 631–636, 2009.

MACFARLANE, R.D.; BULLOCK, G.L.; MCLAUGHLIN, J.J.A. Effects of five metals on susceptibility of striped bass to Flexibacter columnaris. **Transactions of the American Fisheries Society**, v. 115, p. 227–231, 1986.

MACHADO, A.A.; HOFF, M.L.M.; KLEIN, R.D.; CARDOZO, J.G.; GIACOMIN, M.M.; PINHO, G.L.L.; BIANCHINI, A. Biomarkers of waterborne copper exposure in

the guppy *Poecilia vivipara* acclimated to salt water. **Aquatic Toxicology**, v. 138, v. 60–69, 2013.

MANFREDI, G.; YANG, L.; GAJEWSKI, C.D.; MATTIAZZI, M. Measurements of ATP in mammalian cells. **Methods**, v. 26, p. 317-326, 2002.

MANZL, C.; ENRICH, J.; EBNER, H.; DALLINGER, R.; KRUMSCHNABEL, G. Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. **Toxicology**, v. 196, p. 57-64, 2004.

MARAIS, R.; MARSHALL, C.J. Control of the ERK MAP kinase cascade by Ras and Raf. **Cancer Survey**, v. 27, p. 101–125, 1996.

MARTIN, V.; SAINZ, R.M.; MAYO, J.C.; ANTOLIN, I.; HERRERA, F.; RODRIGUEZ, C. Daily rhythm of gene expression in rat superoxide dismutases. **Endocrine Research**, v. 29, p. 83–95, 2003.

MATTIE, M.D.; FREEDMAN, J.H. Copper-inducible transcription: regulation by metal- and oxidative stress responsive pathways. American Journal of Physiology-Cell **Physiology**, v. 286, p. C293-C301, 2004.

MATTIE, M.D.; MCELWEE, M.K.; FREEDMAN, J.H. Mechanism of copper-activated transcription: activation of AP-1, and the JNK/SAPK and p38 signal transduction pathways. **Journal of Molecular Biology**, v. 383, p. 1008-1018, 2008.

MCELWEE, M.K.; SONG, M.O.; FREEDMAN, J.H. Copper activation of NF-κB signaling in HepG2 cells. **Journal of Molecular Biology**, v. 393, p. 1013-1021, 2009.

MELA, M.; GUILOSKI, I.C.; DORIA, H.B.; RABITTO, I.S.; DA SILVA, C.; MARASCHI, A.C.; PRODOCIMO, V.; FREIRA, C.A.; RANDI, M.A.F.; OLIVEIRA RIBEIRO, C.A.; ASSIS, H.C.S. Risks of waterborne copper exposure to a cultivated freshwater Neotropical catfish (*Rhamdia quelen*). **Ecotoxicology and Environmental Safety,** v. 88, p. 108–16, 2013.

MILANI, P.; GALIARDI, S.; COVA, E.; CEREDA, C. SOD1 transcriptional and posttranscriptional regulation and its potential implications in ALS. **Neurology Research International**, v. 2011:9, 2011.

MOORE, H.; WHITMORE, D. Circadian rhythmicity and light sensitivity of the zebrafish brain. **PloS One**, v. 9, p. e86176, 2014.

MRACEK, P.; SANTORIELLO, C.; IDDA, M.L.; PAGANO, C.; BEN-MOSHE, Z.; GOTHILF, Y.; VALLONE, D.; FOULKES, N. S. (2012). Regulation of per and cry Genes Reveals a Central Role for the D-Box Enhancer in Light-Dependent Gene Expression. **PLoS ONE**, v. 7, p. e51278, 2012.

MRACEK, P.; PAGANO, C.; FROHLICH, N.; IDDA, M.L.; CUESTA, I.H.; LOPEZ-OLMEDA, J.F.; SANCHEZ-VAZQUEZ, F.J.; VALLONE, D.; FOULKES, N. S. ERK Signaling Regulates Light-Induced Gene Expression via D-Box Enhancers in a Differential, Wavelength-Dependent Manner. **PLoS ONE**, v. 8, p. 1–10, 2013.

MUKAKA, M.M. Statistics corner: A guide to appropriate use of correlation coefficient in medical research. **Malawi Medical Journal**, v. 24, p. 69–71, 2012.

NADER, N.; CHROUSOS, G. P.; KINO, T. Interactions of the circadian CLOCK system and the HPA axis. **Trends in Endocrinology & Metabolism**, v. 21, p. 277-286, 2010.

NAVARRO, R. F. A evolução dos materiais. Parte 1: da Pré-história ao início da Era Moderna. **REMAP**, v. 1, p. 01-1, 2006.

NGUYEN, T.; SHERRATT, P.J.; PICKETT, C.B. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. **Annual Review of Pharmacology and Toxicology**, v. 43, p. 233-260, 2003.

NORDBERG, J.; ARNÉR, E. S. J. Reactive oxygen species antioxidants and the mammalian thioredoxin system. **Free Radical Biology and Medicine**, v. 31, p. 1287-1311, 2001.

OSTRAKHOVITCH, E.A.; LORDNEJAD, M.R.; SCHLIESS, F.; SIES, H.; KLOTZ, L.O. Copper ions strongly activate the phosphoinositide-3-kinase/Akt pathway independent of the generation of reactive oxygen species. **Archives of Biochemistry and Biophysics**, v. 397 p. 232-239, 2002.

O'NEILL, J.S.; FEENEY, K.A. Circadian Redox and Metabolic Oscillations in Mammalian Systems. **Antioxidants & Redox Signaling**, v. 20, p. 2966–2981, 2014.

PAGANO, C.; CEINOS, R.M.; VALLONE, D.; FOULKES, N.S. **The Fish Circadian Timing System**: The Illuminating Case of Light-Responsive Peripheral Clocks. In: Biological Timekeeping: Clocks, Rhythms and Behaviour (pp. 177-192). Springer, New Delhi, 2017.

PANDO, M.P.; PINCHAK, B.; CERMAKIAN, N.; SASSONE-CORSI, P. A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. **Proceedings of the National Academy of Sciences of the United States of America,** v. 98, p. 10178–10183, 2001.

PANDO, M. P.; SASSONE-CORSI, P. Unraveling the mechanisms of the vertebrate circadian clock: zebrafish may light the way. BioEssays: **News and Reviews in Molecular, Cellular and Developmental Biology,** v. 4, p. 419–26, 2002.

PAQUIN, P.R.; GORSUCH, J.W.; APTE, S.; BATLEY, G.E.; BOWLES, K.C.; CAMPBELL, P.G.C.; DELOS, C.G.; DI TORO, D.M.; DWYER, R.L.; GALVEZ, F.; GENZEMER, R. W.; GOSS, G.G.; HOGSTRAND, C.; JANSSEN, C.R.; PAVANELLI, G.C.; EIRAS, J.C.; TAKEMOTO, R.M. **Doenças de peixes: profilaxia diagnóstico e tratamento**, 2ed. Eduem, Maringá, 2002.

PARK, E.Y.; RHO, H.M. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. **Molecular and Cellular Biochemistry**, v. 240, p. 47-55, 2002.

PATEL, S.; VELINGKAAR, N. S.; KONDRATOV, R. V. Transcriptional control of antioxidant defense by the circadian clock. **Antioxidants & Redox Signaling**. v. 20, p. 2997–3006, 2014.

PEDROZO, M. F. M.; LIMA, I. V. **Ecotoxicologa do cobre e seus compostos**. In Série Cadernos de Referência Ambiental V. 2. Salvador, 2001.

PEYRIC, E.; MOORE, H.A.; WHITMORE, D. Circadian clock regulation of the cell cycle in the zebrafish intestine. **PloS One**, v. 8, p. e73209, 2013.

PHAM, A.N.; XING, G.; MILLER, C.J.; WAITE, T.D. Fenton-like copper redox chemistry revisited: Hydrogen peroxide and superoxide mediation of copper-catalyzed oxidant production. **Journal of Catalysis**, v. 301, p. 54-64, 2013

PLAUTZ, J. D.; KANEKO, M.; HALL, J. C.; KAY, S. A. Independent photoreceptive circadian clocks throughout Drosophila. **Science**, v. 278, p. 1632–1635 1997.

QU, R.; FENG, M.; WANG, X.; QIN, L.; WANG, C.; WANG, Z.; WANG, L. Metal accumulation and oxidative stress biomarkers in liver of freshwater fish *Carassius auratus* following in vivo exposure to waterborne zinc under different pH values. **Aquatic Toxicology**, v. 150, p. 9–16, 2014.

QIAN, Y.; KHATTAK, S.F.; XING, Z.; HE, A.; KAYNE, P.S.; QIAN, N.X.; PAN, S.; LI, Z. J. Cell culture and gene transcription effects of copper sulfate on Chinese hamster ovary cells. **Biotechnology Progress**, v. 27, p. 1190-1194, 2001.

QIAN, J.; BLOCK, G.D.; COLWELL, C.S.; MATVEYENKO, A.V. Consequences of exposure to light at night on the pancreatic islet circadian clock and function in rats. **Diabetes**, v. 62, p. 3469-3478, 2013.

RAMOS, B.C.; MORAES, M.N.C.; POLETINI, M.O.; LIMA, L.H.; CASTRUCCI, A.M.L. From blue light to clock genes in zebrafish ZEM-2S cells. **PLoS One**, v. 9, p. e106252, 2014.

RAU, M.A.; WHITAKER, J.; FREEDMAN, J.H.; DI GIULIO, R. T. Differential susceptibility of fish and rat liver cells to oxidative stress and cytotoxicity upon exposure to prooxidants. **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,** v. 137, p. 335-342, 2004.

RAVINDRAN, J.; GUPTA, N.; AGRAWAL, M.; BHASKAR, A.B.; RAO, P.L. Modulation of ROS/MAPK signaling pathways by okadaic acid leads to cell death via, mitochondrial mediated caspase-dependent mechanism. **Apoptosis**, v. 16, p. 145-161, 2011.

REPPERT, S. M.; WEAVER, D. R. Coordination of circadian timing in mammals. **Nature**, v. 418, p. 935–941, 2002.

RHEE, J.S.; KIM, B.M.; LEE, B.Y.; HWANG, U.K.; LEE, Y.S.; LEE, J.S. Cloning of circadian rhythmic pathway genes and perturbation of oscillation patterns in endocrine disrupting chemicals (EDCs)-exposed mangrove killifish *Kryptolebias marmoratus*. **Comparative Biochemistry and Physiology. Toxicology & Pharmacology**, v. 164, p. 11–20, 2014.

ROBINS, R.G.; BERG, R.B.; DYSINGER, D.K.; DUAIME, T.E.; METESH, J.J.; DIEBOLD, F.E.; TWIDWELL L.; MITMAN, G.; CHATHAM, W.; HUANG, H.; YOUNG, C.A**. Chemical, physical and biological interaction at the Berkeley Pit, Butte, Montana. Tailings and Mine Waste**. v.97, pp. 529-541. Rotterdam, The Netherlands: Bakeman. 1997.

RUTTER, J.; REICK, M.; MCKNIGHT, S.L. Metabolism and the control of circadian rhythms. **Annual Review of Biochemistry**, v. 71, p. 307–331, 2002.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. **Molecular cloning: a laboratory manual** (Ed. 2). Cold spring harbor laboratory press. 1989

SAMET, J.M.; GRAVES, L.M.; QUAY, J.; DAILEY, L.A.; DEVLIN, R.B.; GHIO, A.J.; WU, W.; BROMBERG, P.A.; REED, W. Activation of MAPKs in human bronchial epithelial cells exposed to metals. **American Journal of Physiology-Lung Cellular and Molecular Physiology,** v. 275, p. L551–L558, 1998.

SANDRINI, J.Z.; BIANCHINI, A.; TRINDADE, G.S.; NERY, L.E.M.; MARINS, L.F.F. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish (*Danio rerio*) ZFL cells. **Aquatic Toxicology**, v. 95, p. 285-291, 2009.

SANI, M.; SEBAI, H.; GADACHA, W.; BOUGHATTAS, N.; REINBERG, A.; MOSSADOCK, B.A. Catalase activity and rhythmic patterns in mouse brain, kidney and liver. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, v. 145, p. 331-337, 2006.

SANTOS, E.M.; BALL, J.S.; WILLIAMS, T.D.; WU, H.; ORTEGA, F.; VAN AERLE, R.; KATSIADAKI, I.; FALCIANI, F.; VIANT, M.R.; CHIPMAN, J.K.; TYLER, C.R.

Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. **Environmental Science & Technology**, v. 44, p. 820-826, 2009.

SHI, X.; ZHOU, B. The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. **Toxicological Sciences**, v. 115, p. 391-400, 2010.

SIMONATO, J. D.; MELA, M.; DORIA, H. B.; GUILOSKI, I. C.; RANDI, M. A. F.; CARVALHO, P. S.; MELETTI, P. C.; SILVA DE ASSIS, H. C.; BIANCHINI, A.; MARTINEZ, C. B. R. Biomarkers of waterborne copper exposure in the Neotropical fish *Prochilodus lineatus*. **Aquatic Toxicology**, v. 170, p. 31-41, 2015.

SILVA ACOSTA, D.; DANIELLE, N.M.; ALTENHOFEN, S.; LUZARDO, M.D.; COSTA, P.G.; BIANCHINI, A.; BONAN, C.D.; SILVA, R.S.; DAFRE, A.L. Copper at low levels impairs memory of adult zebrafish (*Danio rerio*) and affects swimming performance of larvae. **Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology**, v. 185, p. 122–130, 2016.

SLOMAN K.A.; SCOTT, G.R.; DIAO, Z.; ROULEAU, C.; WOOD, C.M.; MCDONALD, D.G. Cadmium affects the social behaviour of rainbow trout, *Oncorhynchs mykiss*. **Aquatic Toxicology**, v. 65, p. 171-185, 2003.

SON, Y.; CHEONG, Y.K.; KIM, N.H.; CHUNG, H.T.; KANG, D.G.; PAE, H.O. Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? **Journal of Signal Transduction**, v. 2011, p. 792639, 2011.

SONG, M.O.; LI, J.; FREEDMAN, J.H. Physiological and toxicological transcriptome changes in HepG2 cells exposed to copper. **Physiological Genomics**, v. 38, p. 386-401, 2009.

STORZ, P. Forkhead homeobox type O transcription factors in the responses to oxidative stress. **Antioxidants & Redox Signaling**. v. 14, p. 593-605, 2011.

STRAUS, D.L.; TUCKER, C.S. Acute toxicity of copper sulfate and chelated copper to channel catfish *Ictalurus puntatus*. **Journal of Aquaculture Society. Baton Rouge**, v. 24, p. 390-395, 1993.

SUBRAMANIAN, P.; DAKSHAYANI, K.B.; PANDI-PERUMAL, S.R.; TRAKHT, I.; CARDINALI, D.P. 24-Hour Rhythms in Oxidative Stress During Hepatocarcinogenesis in Rats: Effect of Melatonin or Alpha-Ketoglutarate. Redox Report**: Communications in Free Radical Research**, v. 13, p. 78–86, 2008.

TAMAI, T.K.; YOUNG, L.C.; WHITMORE, D. Light signaling to the zebrafish circadian clock by Cryptochrome 1a. **Proceedings of the National Academy of Sciences of the United States of America**, v. 104, p. 14712–14717, 2007.

TAN, F.; WANG, M.; WANG, W.; LU, Y. Comparative evaluation of the cytotoxicity sensitivity of six fish cell lines to four heavy metals in vitro. **Toxicology in vitro**, v. 22, p. 164-170, 2008.

TISCHKAU, S.A.; MITCHELL, J.W.; TYAN, S.H.; BUCHANAN, G.F.; GILLETTE, M.U. Ca^{2+}/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. **Journal of Biological Chemistry**, v. 278, p. 718-723, 2003.

TOMÁS-ZAPICO, C.; COTO-MONTES, A.; MARTÍNEZ-FRAGA, J.; RODRÍGUEZ-COLUNGA, M.J.; TOLIVIA, D. Effects of continuous light exposure on antioxidant enzymes, porphyric enzymes and cellular damage in the Harderian gland of the Syrian hamster. **Journal of Pineal Research**, v. 34, p. 60-68, 2003.

TSENG, H.L.; LI, C.J.; HUANG, L.H.; CHEN, C.Y.; TSAI, C.H.; LIN, C.N.; HSU, H.Y. Quercetin 3-O-methyl ether protects FL83B cells from copper induced oxidative stress through the PI3K/Akt and MAPK/Erk pathway. **Toxicology and Applied Pharmacology**, v. 264, p. 104-113, 2012.

TURSKI, M.L.; BRADY, D.C.; KIM, H.J.; KIM, B.E.; NOSE, Y.; COUNTER, C.M.; THIELE, D.J. A novel role for copper in Ras/mitogen-activated protein kinase signaling. **Molecular and Cellular Biology**, v. 32, p. 1284-1295, 2012.

US EPA- United States Environmental Protection Agency. **Ambiental water quality criteria for copper**. Washington, DC. 1984.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environmental Toxicology and Pharmacology**, v. 13, p. 57–149, 2003.

VALKO, M.; MORRIS, H.; CRONIN, M.T.D. Metals, Toxicity and oxidative stress. **Current Medicinal Chemistry**. v. 12, p. 1161-1208, 2005.

VALLONE, D.; GONDI, S.B.; WHITMORE, D.; FOULKES, N.S. E-box function in a period gene repressed by light. **Proceedings of the National Academy of Sciences of the United States of America,** v. 101, p. 4106–4111, 2004.

VALLONE, D.; SANTORIELLO, C.; GONDI, S.B., FOULKES, N.S. **Basic Protocols for Zebrafish Cell Lines**. In Circadian Rhythms (pp. 429-441). Humana Press. 2007.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review**. Environmental Toxicology and Pharmacology**, v. 13, p. 57-149, 2003.

VAN TIEM, L.A.; DI GIULIO, R. T. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE-and ARE-associated gene induction in zebrafish (*Danio rerio*). **Toxicology and Applied Pharmacology**, v. 254, p. 280-287, 2011.

VARO, I.; et al. Effect of sublethal concentrations of copper sulphate on seabream *Sparus aurata* fingerlings. **Aquatic Living Resources**, v. 20, p. 263-270, 2007.

VATINE, G.; VALLONE, D.; APPELBAUM, L.; MRACEK, P.; BEN-MOSHE, Z.; LAHIRI, K.; GOTHILF, Y.; FOULKES, N.S. Light directs zebrafish period2 expression via conserved D and E boxes. **PLoS Biol**, v. 7, p. e1000223, 2009.

VATINE, G.; VALLONE, D.; GOTHILF, Y.; FOULKES, N. S. It's time to swim! Zebrafish and the circadian clock. **FEBS Letters**, v. 585, p. 1485–1494, 2011.

VELARDEA, E.; HAQUEB, A.; IUVONEB, P.M.; AZPELETA, C.; ALONSOGÓMEZ, A.L.; DELGADO, M.J.D. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of Period and Cryptochrome transcripts in retina, liver, and gut. **Journal of Biological Rhythms,** v. 24, p. 104–113, 2010.

VERA, L.M.; MIGAUD, H. Hydrogen peroxide treatment in Atlantic salmon induces stress and detoxification response in a daily manner. **Chronobiology International**, v. 33, p. 530-542, 2016.

VIEIRA, L.R.; GRAVATO, C.; SOARES, M.V.M.; MORGADO, F.; GUILHERMINO, L. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. **Chemosphere,** v. 76, p. 1416–1427, 2009.

VOLPATO, G. L.; TRAJANO, E. Biological rhythms. In: **Fish Physiology**: The Physiology of Tropical Fishes: The Physiology of Tropical Fishes. p.21-101. 2005.

WANG, B.; FENG, L.; JIANG, W.D.; WU, P.; KUANG, S.Y.; JIANG, J.; TANG, L.; TANG, W.N.; ZHANG, Y.A.; LIU, Y.; ZHOU, X.Q. Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: preventive role of arginine. **Aquatic Toxicology**, v. 158, p. 125-137, 2015.

WANG, H. Comparative analysis of teleost fish genomes reveals preservation of different ancient clock duplicates in different fishes. **Marine Genomics**, v. 1, p. 69–78, 2008.

WANG, X.; TOMSO, D.J.; CHORLEY, B.N.; CHO, H.Y.; CHEUNG, V.G.; KLEEBERGER, S.R.; BELL, D.A. Identification of polymorphic antioxidant response elements in the human genome. **Human Molecular Genetics**, v. 16, p. 1188-1200, 2007.

WANG, X.J.; HAYES, J.D.; HIGGINS, L.G.; WOLF, C.R.; DINKOVA-KOSTOVA, A.T. Activation of the NRF2 signaling pathway by copper-mediated redox cycling of para-and ortho-hydroquinones. **Chemistry & Biology**, v. 17, p. 75-85, 2010.

WEGER, B.D.; SAHINBAS, M.; OTTO, G.W.; MRACEK, P., ARMANT, O., DOLLE, D.; LAHIRI, K.; VALLONE, D.; ETTWILLER, L.; GEISLER, R.; FOULKES, N.S.; DICKMEIS, T. The light responsive transcriptome of the Zebrafish: Function and regulation. **PLoS ONE**, v. 6, p. e17080, 2011.

WENDELAAR BONGA, S. E. The Stress Response in Fish. **Physiological Reviews**, v. 77, p. 591- 620, 1997.

WHITMORE, D.; FOULKES, N. S.; SASSONE-CORSI, P. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. **Nature**, v. 404, p. 87–91, 2000.

WILKING, M.; NDIAYE, M.; MUKHTAR, H.; AHMAD, N. Circadian rhythm connections to oxidative stress: implications for human health. **Antioxidants & Redox Signaling**, v. 19, p. 192-208, 2013.

WU, L.; REDDY, A.B. Rethinking the clockwork: redox cycles and non-transcriptional control of circadian rhythms. **Biochemical Society Transactions**, v. 42, p. 1–10, 2014.

XU, Y.Q.; ZHANG, D.; JIN, T.; CAI, D.J.; WU, Q.; LU, Y.; LIU, J.; KLAASSEN, C.D. Diurnal Variation of Hepatic Antioxidant Gene Expression in Mice. **PLoS ONE,** v. 7, p. e44237, 2012.

YIN, J.; WANG, A.P.; LI, W.F.; SHI, R.; JIN, H.T.; WEI, J.F. Time-response characteristic and potential biomarker identification of heavy metal induced toxicity in zebrafish. **Fish & Shellfish Immunology**, v. 72, p. 309-317, 2018.

ZAVITSANOS, K.; NUNES, A.M.; MALANDRINOS, G.; HADJILIADIS, N. Copper effective binding with 32–62 and 94–125 peptide fragments of histone H2B. **Journal of Inorganic Biochemistry,** v. 105, p. 102-110, 2011.

ZELINSKI, E. L.; DEIBEL, S. H.; MCDONALD, R. J. The trouble with circadian clock dysfunction: multiple deleterious effects on the brain and body. **Neuroscience and Behavioral Reviews**, v. 40, p. 80–101, 2014.

ZELKO, I.N.; MARIANI, T.J.; FOLZ, R.J. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. **Free Radical Biology and Medicine**, v. 33, p. 337-349, 2002.

ZHOU, J.; ZHU, X.S.; CAI, Z.H. The impacts of bisphenol A (BPA) on abalone (*Haliotis diversicolor supertexta*) embryonic development. **Chemosphere**, v. 82, p. 443-450, 2011.

ZIV, L.; LEVKOVITZ, S.; TOYAMA, R.; FALCON, J.; GOTHILF, Y. Functional development of the zebrafish pineal gland: Light-induced expression of period2 is required for onset of the circadian clock. **Journal of Neuroendocrinology**, v. 17, p. 314–320, 2005.

9. RESULTADOS ADICIONAIS

CATALASE – Expressão endógena em DD e em LD após exposição ao cobre e estudo da sequência clonada no repórter de luciferase.

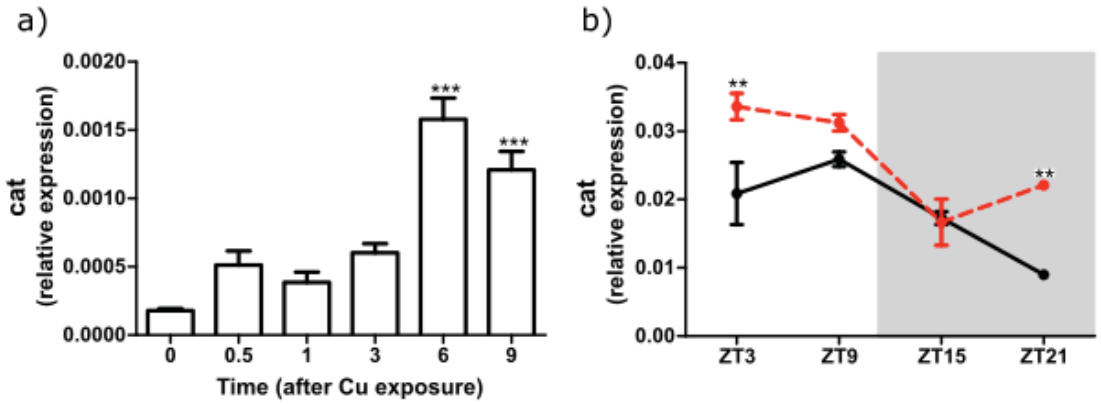


Fig A. a) qRT-PCR analysis of the expression of the cat gene under DD conditions treated with 250 µM Cu. Relative mRNA levels are plotted on the y-axis and time in hours after the Cu exposure on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Asterisks indicate effects in comparison to control "0" (***p<0.001). b) qRT-PCR analysis of the expression of the cat gene in PAC-2 entrained in LD conditions treated with 250 µM Cu (red line) or negative control treated with vehicle (black). Relative mRNA levels are plotted on the y-axis and ZT times on the x-axes. The results are plotted as the means of three independent experiments +/− SD. Asterisks indicate difference in comparison to control (**p<0.01).

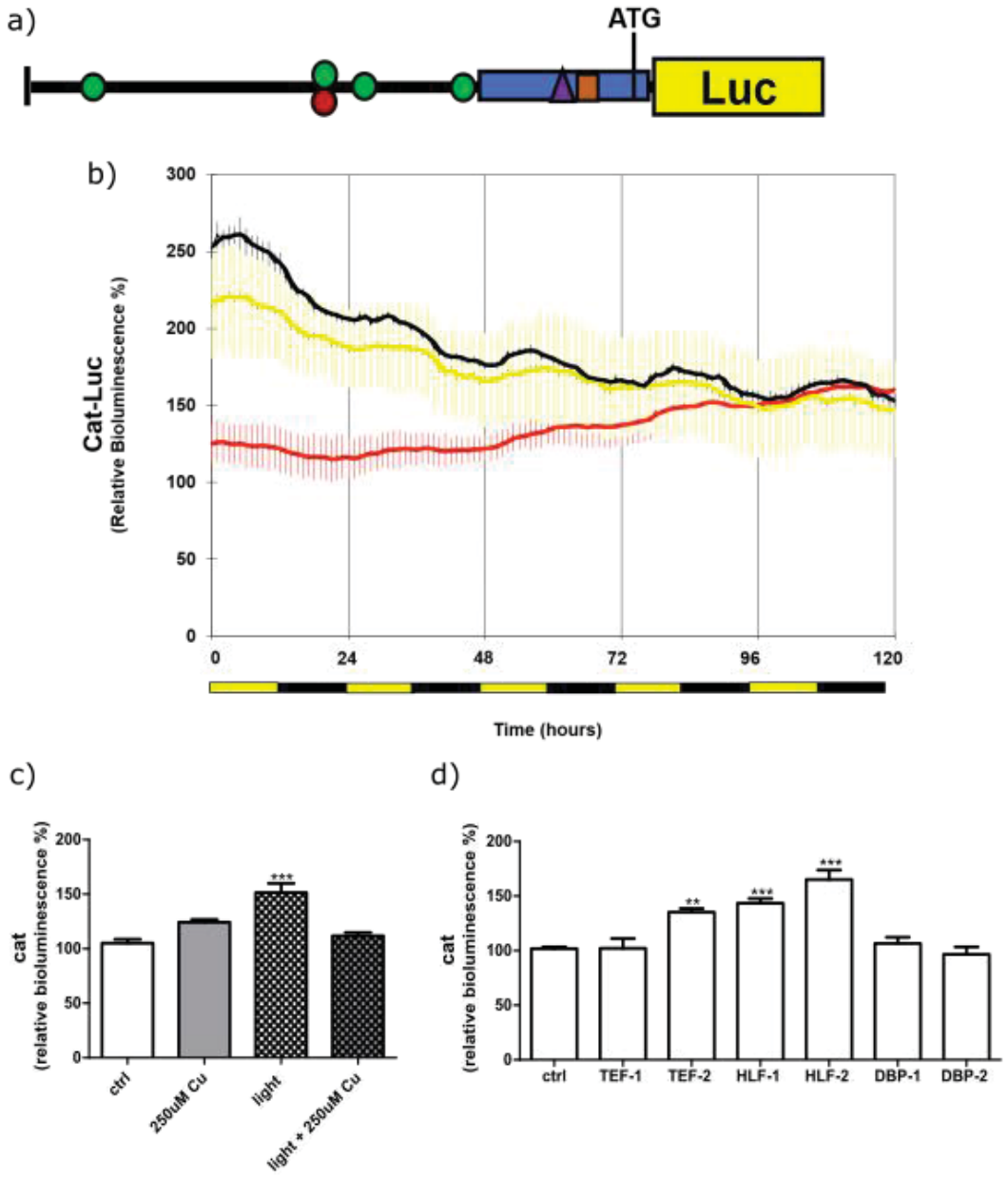


Fig B. a) Representative figure of the cloned cat promoter and the enhancer elements: TEF, VBP, HLF (green circle), E4BP4 (red circle), ARE (brown square) and E-box (purple triangle). b) Real time bioluminescence assays of PAC-2 cells transfected with Cat-Luc treated with 250 µM Cu (red line), 300 µM of H_2O_2 (yellow line) or a negative control treated with vehicle (black line). The start of treatments happened shortly before time zero. Bioluminescence is plotted on the y-axis and time (hours) on the x-axis. Each time-point represents the mean of at least three independent samples. Black and yellow bars below the panels represent the different lighting conditions. c) In vitro luciferase assay of PAC-2 cells transfected with the Cat-Luc reporter. Cells were left in DD conditions for 2 days before exposure to 8 hours of constant light and/or 32 hours of 250 µM of Cu; each exposure regime is indicated below its respective bars. Relative bioluminescence levels (%) are plotted on the y-axis where the highest value measured during the experiment is set arbitrarily as 100%. The results are plotted as the means of three independent experiments performed in triplicate, +/− SD. Each independent experiment was standardized for transfection efficiency using a β-galactosidase assay. Asterisks indicate effects in comparison to control (***$p<0.001$). d) In vitro luciferase

assay of PAC-2 cells co-transfected with expression constructs encoding the six PAR/bZip factors and the Cod-Luc reporter. Cells were left in DD conditions for 2 days after the co-transfection and then sampled. Each expression construct is indicated below its respective bars. Relative bioluminescence levels (%) are plotted on the y-axis where the highest value measured during the experiment is set arbitrarily as 100%. The results are plotted as the means of three independent experiments performed in triplicate, +/− SD. Each independent experiment was standardized for transfection efficiency using a β-galactosidase assay. Asterisks indicate effects in comparison to control (**$p<0.01$, ***$p<0.001$).